# Ability of Poly-L-arginine to Enhance Drug Absorption into Aqueous Humor and Vitreous Body after Instillation in Rabbits

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> The effect of poly-L-arginine with a molecular weight of 35.5 kDa (PLA) on the ocular absorption of hydrophilic molecules after instillation was examined in rabbits *in vivo*. FITC-labeled dextran (3.8 kDa, FD-4) and pyridoxamine were used as model hyprophilic molecules for absorption. The potential toxicity of PLA was evaluated by microscopic observation of the cornea, production of TNF-$\alpha$, and the thickness of the corneal epithelia and stroma. The concentration of pyridoxamine and FD-4 in aqueous humor 30 min after a single instillation of a solution of PLA was 29- and 16-fold higher than that without PLA, respectively, but the drug concentrations were not determined in the vitreous body. Repetitive instillation of PLA every 30 min for 150 min achieved 31.1- and 13.3-fold increases in pyridoxamine and FD-4 in aqueous humor, respectively. Furthermore, significant amounts of pyridoxamine and FD-4 were detected in the vitreous body after the repetitive instillation of PLA, even although very little of these drugs was detected in the vitreous body in the control eye without PLA. On the other hand, repetitive instillation of PLA did not induce any alteration of corneal epithelial and stromal thickness, production of TNF-$\alpha$, and disruption of the epithelial and stromal morphologies and neutrophil infiltration. Our findings suggest that PLA may be useful in promoting drug delivery of hydrophilic drugs to the ocular tissues without producing any significant corneal damage and inflammation.

> **Key words**    poly-L-arginine; absorption enhancer; instillation; inflammation

Instillation is the drug administration method most commonly used in pharmacotherapy in ophthalmology as far as convenience and safety are concerned. However, absorption of drugs after instillation is restricted because of the barrier properties of surface ocular tissues, such as the cornea and conjunctiva. Use of penetration enhancers is one means of improving such restrictions.[1] From a safety point of view, it is important to maintain the viability of the living epithelial cells after application of the enhancers. Among the penetration enhancers studied previously, cationic polymers such as chitosan, aminated gelatin, and poly-L-arginine are reported to increase the transepithelial absorption of peptide drugs by dissociation of tight junction assemblies which restrict the paracellular permeation in intestinal and nasal epithelia without producing significant epithelial damage.[2—6] Thus, polycationic polymers may be useful penetration enhancers for ocular drug delivery.

In a previous *in vitro* study,[7] we showed that poly-L-arginine (PLA), a cationic polymer, with a molecular weight range of 14.0—141.4 kDa, increased the epithelial transport of pyridoxamine (MW: 241.1) and FITC-labeled dextran (MW *ca.* 3.8 kDa, FD-4) through the excised cornea, conjunctiva, and conjunctiva/sclera composite by changing paracellular permeability. Because the PLA-induced recovered after removal of PLA, similar to that reported in the nasal epithelium[8,9] and PLA did not affect the cellular viability as depicted by MTT assay,[7] PLA can be used as an absorption enhancer for ocular drug delivery. However, until now, the *in vivo* enhancing ability and potential toxicity of PLA after instillation have not been examined.

The purpose of the present study was to evaluate the effect of PLA on the intraocular absorption of pyridoxamine and FD-4 and the potential toxic effects after instillation of PLA

solution in rabbits. PLA with a molecular weight of 35.5 kDa was chosen for the present study. Drug concentrations in aqueous humor and vitreous body was measured after single and multiple instillations of PLA solution to evaluate the *in vivo* enhancing ability. The potential toxicity was evaluated by microscopic observation of the cornea, production of TNF-$\alpha$, and thickness of the corneal epithelia and stroma which is used as an index of corneal inflammation.[10,11]

## MATERIALS AND METHODS

**Materials**   Poly-L-arginine hydrochloride (MW *ca.* 35.5 kDa, PLA), fluorescein isothiocyanate-dextran (MW *ca.* 3.8 kDa, FD-4), lipopolysaccharides (LPS) from *Pseudomonas aeruginosa* 10, leupeptin, sodium orthovanadate, phenylmethanesulfonyl fluoride, pepstatin A, approtinin, and benzamidine were purchased from Sigma Chemical Co., Ltd. (St. Louis, MO, U.S.A.). Mayer's hematoxylin and eosin were obtained from Sakura Finetek Japan Co., Ltd. (Tokyo, Japan). Pyridoxamine was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Sodium taurodihydrofusidate (STDHF) was supplied by Leo Pharmaceuticals (Ballerup, Denmark). Goat anti-rabbit TNF-$\alpha$ polyclonal antibody and horseradish peroxidase conjugated donkey anti-goat IgG antibody were obtained from BD Biosciences (San Jose, CA, U.S.A.) and Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, U.S.A.), respectively. All other compounds were of reagent grade.

**Animals**   Male Japanese white rabbits (Tokyo Laboratory Animals, Tokyo, Japan), weighing 1.8 to 2.2 kg were sacrificed by slowly increasing the $CO_2$ concentration in a $CO_2$ gas animal euthanasia cabinet (KN-750-1, Natsume Co., Ltd., Tokyo, Japan) according to the NIH standards as de-

scribed in "Principles of Laboratory Animal Care."

**Drug Administration** In a single instillation study, 50 $\mu$l bicarbonate Ringer's solution (BRS, composed of 111.5 mM NaCl, 4.8 mM KCl, 29.2 mM NaHCO$_3$, 0.75 mM NaH$_2$PO$_4$, 1.04 mM CaCl$_2$, 0.74 mM MgCl$_2$, and 5 mM D-glucose) containing either FD-4 or pyridoxamine solution at 2.5 mg/ml was instilled into the cul-de-sac of the left eye of the rabbit (control eye). The drug solution containing PLA at 5 mg/ml was instilled into the other eye (test eye). At 10, 30, or 60 min after instillation, rabbits were sacrificed and aqueous humor and vitreous body were then immediately taken from each eye. The aqueous humor was filtered through a Chromatodisk (0.45 $\mu$m, Cosmo Bio Co., Ltd., Tokyo, Japan), then centrifuged at 12000 $\boldsymbol{g}$ for 10 min at 4 °C. The resulting supernatant was subjected to HPLC. The vitreous body was centrifuged at 12000 $\boldsymbol{g}$ for 10 min at 4 °C, then the resulting supernatant was mixed with a similar volume of acetonitrile. After mixing, the solution was centrifuged at 12000 $\boldsymbol{g}$ for 10 min at 4 °C, and the resulting supernatant was subjected to HPLC.

In the multiple instillation study, the left eye was served as the control eye and the right eye as the test eye as described above. Instillations with the solutions described above were performed 5 times at 30 min intervals. Rabbits were sacrificed at 30 min after the final instillation and aqueous humor and vitreous body were immediately collected from each eye for drug assay using HPLC. The area under the aqueous humor concentration–time curve up to 60 min ($AUC_{0-60}$) was calculated by the trapezoidal method.

**HPLC Conditions** Pyridoxamine and FD-4 were determined by HPLC. The HPLC system consisted of a system controller (SCL-10A$_{VP}$, Shimadzu, Kyoto, Japan), auto injector (SIL-10A$_{XL}$, Shimadzu), pump (LC-10AT$_{VP}$, Shimadzu), degasser (DGU-12A, Shimadzu), column oven (CTO-10A, Shimadzu), fluorescence spectrophotometer (RF-10A$_{XL}$, Shimadzu), and spectrophotometer (RF-10A$_{XL}$, Shimadzu). A separation column of TOSHO TSK-GEL octadecyl-NPR (3.5 cm×4.6 mm i.d., 2.5 $\mu$m, Tosho Co., Tokyo, Japan) was used with a mobile phase consisting of 10 mM phosphate buffer (pH 8.0) : acetonitrile (90 : 10) for the analysis of FD-4. The flow rate was 1.0 ml/min. The excitation and emission wavelengths for detection were 488 nm and 515 nm, respectively. Pyridoxamine was measured as described by Bisp *et al.* with minor modifications.[12] Mightysil RP-18 (25 cm× 4.6 mm I.D., 5 mm, Kanto Chemical Co., Inc., Tokyo, Japan) was used as the separation column. Mobile phase A (3.5 mM 1-octanesulfonic acid and 1.2 mM triethylamine in 0.033 M phosphoric acid) and mobile phase B (acetonitrile) were mixed under gradient conditions of A : B (15 : 85) to A : B (30 : 70) from 0 to 14 min. The flow rate was 1.2 ml/min. K$_2$HPO$_4$ buffer (770 mM) containing 5.26 mM sodium bisulfite was used as the post-column reagent, and the flow rate was 0.3 ml/min. Excitation and emission wavelengths for detection were 328 nm and 393 nm, respectively.

**Histological Observations** The potential induction of corneal inflammation was assessed by microscopic observation. STDHF, a bile salt derivative, which is known to induce severe epithelial damage[10,11] and LPS from *Pseudomonas aeruginosa* which induces keratitis[13,14] were used as positive controls in this study. The left eye of each rabbit was used as a negative control and treated with BRS alone (50 $\mu$l) 3 times

a day (a.m. 9:00, p.m. 3:00 and p.m. 9:00) for 5 d. In the case of the enhancer treatment, 50 $\mu$l of the solution containing either PLA or STDHF at 5 mg/ml was instilled into the right eye 3 times a day for 5 d. In the LPS study, the corneal epithelium was abraded with a modified 26-gauge needle and 100 $\mu$g LPS was administered to the ocular surface, as described in a previous report of a keratitis model.[13] Each eye ball was excised from the orbit 30 min after the final instillation and the entire eye ball was fixed with 2.5% glutaraldehyde/TBS-Ca buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 10 mM CaCl$_2$) for 1 min, rinsed in TBS-Ca buffer, and embedded in 4% carboxymethyl cellulose (Finetec Co., Ltd., Tokyo, Japan). Cryosections (*ca.* 5 $\mu$m) were prepared with a Leica CM3050S cryostat (Leica, Nussloch, Germany). Then, sections were stained with hematoxylin and eosin. The specimen was observed under a microscope (BX51WI; Olympus Co., Tokyo, Japan). Visualization and measurement of the corneal thickness were performed with Lumina Vision equipment (Mitani Co., Fukui, Japan).

**Western Blot Analysis** The eye ball treated with either PLA, STDHF, or LPS described above was homogenized in lysis buffer composed of 20 mM HEPES, pH 7.0, 150 mM NaCl, 1.5 mM MgCl$_2$, 1% SDS, 2 $\mu$g/ml leupeptin, 2 mM sodium orthovanadate, 4 mM phenylmethanesulfonyl fluoride, 1 mM pepstatin A, 10 $\mu$g/ml approtinin, and 5 mM benzamidine on ice. Then, the homogenate was centrifuged at 12000 $\boldsymbol{g}$ for 10 min at 4 °C, and the total protein concentration in the supernatant was measured using a BCA protein assay reagent kit (Pierce Biotechnology, Inc., Rockfold, U.S.A.). The total protein concentration in each sample was calibrated with regard to the identical concentration with lysis buffer. Separated supernatant (20 $\mu$l) was mixed with the same volume 2× sample buffer (0.125 M Tris–HCl, pH 6.8, 10% 2-Mercaptoethanol, 4% SDS, 10% Sucrose, 0.004% Bromophenol blue) and boiled at 100 °C for 5 min. SDS-PAGE was performed according to Laemmli's method.[15] For immunoblotting, proteins separated by SDS-PAGE were transferred to PVDF membranes, which were then incubated with Goat anti-rabbit TNF-$\alpha$ antibody (1 : 500) and horseradish peroxidase-conjugated donkey anti-goat IgG antibody (1 : 5000). The signal derived from TNF-$\alpha$ was detected using Super Signal® West Dura Extended Duration Substrate (Pierce Biotechnology, Inc., Rockfold, U.S.A.) by an Image Reader LAS-1000 system (Fuji Photo Film Co., Ltd., Tokyo, Japan).

## RESULTS

**Absorption Enhancing Effect of PLA into Intraocular Tissues** In this study, single and multiple instillations were performed to estimate the absorption enhancing effect of PLA for intraocular delivery of drugs. Figures 1a and b show the concentration of pyridoxamine and FD-4 in aqueous humor, respectively, at 10, 30 and 60 min after a single instillation. The concentration of pyridoxamine in aqueous humor 10 min after a single instillation was 0.01 $\mu$g/ml, while that of FD-4 was below the quantitation limit (200 pg/ml). The peak concentration in aqueous humor was observed at 30 min in both control and PLA groups. The concentration with PLA was 29- and 16-fold higher than that without PLA for pyridoxamine and FD-4, respectively. Furthermore, applica-

tion of PLA increased the $AUC_{0—60}$ by 28-fold for pyridoxamine and 18-fold for FD-4 (Table 1), indicating that a single dose of PLA can improve the bioavailability of drugs as far as the anterior chamber of the eye is concerned. On the other hand, pyridoxamine and FD-4 were not determined in the vitreous body after a single instillation with PLA for at least up to 60 min.

The multiple instillation experiment was conducted to investigate the effect of PLA on drug delivery to both the anterior chamber and posterior segment of the eye. The concentration of pyridoxamine and FD-4 in aqueous humor was increased by 31.1-fold for pyridoxamine and 13.3-fold for FD-4 at 30 min after 5 repeated instillations at 30 min intervals compared with the control of 0.09 and 0.04 $\mu$g/ml for pyridoxamine and FD-4, respectively (Table 2). Repeated application of PLA also increased the concentration of pyridoxamine in vitreous body 5.4-fold compared to that without PLA. Even although no FD-4 was detected in the vitreous body after repeated application of FD-4 alone, a significant concentration of FD-4 (14 ng/ml) appeared in the vitreous body after repeated application of PLA. Thus, PLA appears to have the ability to increase drug absorption into the posterior segment of the eye.

**Change in the Corneal Epithelial and Stromal Thickness** Table 3 shows the thickness of the corneal epithelium and stroma following application of PLA, STDHF, and LPS 3 times a day for 5 d. No significant differences in epithelial and stromal thickness in the cornea were observed between BRS (control) and PLA treatment. On the other hand, STDHF, which was shown to induce epithelial damage to the cornea and the conjunctiva in our previous study,[5] signifi-

cantly reduced the corneal epithelial thickness but not the corneal stromal thickness. LPS treatment, conducted as a positive control for an inflammatory response, significantly reduced the epithelial thickness, but increased the stromal thickness compared with the effects of BRS treatment.

**Observation of Inflammatory Responses** Figures 2a—d show the microscopic images of the corneal stroma following treatment with BRS (control), PLA, STDHF, and LPS 3 times a day for 5 d. LPS induced obvious disruption of the epithelial and stromal morphology and neutrophil infiltration (Fig. 2d). In contrast, no inflammatory responses were observed after treatment with BRS, PLA and STDHF (Figs. 2a—c).

Production of tumor necrosis factor-$\alpha$ (TNF-$\alpha$), a pro-inflammatory cytokine, was detected using Western blot analyses (Fig. 3). TNF-$\alpha$ production was found after treatment with LPS, while no detectable amounts of TNF-$\alpha$ were observed with BRS, PLA, and STDHF, which is consistent with the above microscopic observations.

## DISCUSSION

In the present study, an *in vivo* instillation experiment was conducted to investigate the effect of PLA on drug absorption into the intraocular tissues using hydrophilic model compounds FD-4 and pyridoxamine. In addition, we assessed the potential safety of PLA in terms of inflammatory responses based on the corneal thickness, production of TNF-$\alpha$, and histological observations.

When pyridoxamine and FD-4 without PLA were instilled into the rabbit eye, the maximal concentration of pyridoxamine (0.045 $\mu$g/ml) and FD-4 (0.006 $\mu$g/ml) in aqueous humor was achieved 30 min after a single instillation (Fig. 1). The observed kinetic pattern was consistent with those obtained in previous studies after single instillations of bunazosin,[16]
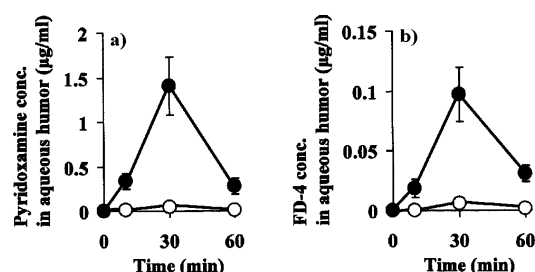


Fig. 1. Concentration–Time Profiles of Pyridoxamine (a) and FD-4 (b) after a Single Instillation in the Presence or Absence of 5 mg/ml PLA (50)

○, in the absence of PLA; ●, in the presence of PLA.

Table 1. $AUC_{0—60}$ of Pyridoxamine and FD-4 in Aqueous Humor Estimated after a Single Instillation

| | Control (min·$\mu$g/ml) | With PLA (min·$\mu$g/ml) |
|---|---|---|
| Pyridoxamine | 1.61 | 44.4 [27.6] |
| FD-4 | 0.18 | 3.18 [17.7] |

[ ]: enhancement ratio *versus* the control.

Table 2. Concentration of Pyridoxamine and FD-4 in Aqueous Humor and Vitreous Body Obtained after Repeated Instillation

| | Aqueous humor ($\mu$g/ml) | | Vitreous body ($\mu$g/ml) | |
|---|---|---|---|---|
| | Control | With PLA | Control | With PLA |
| Pyridoxamine | 0.09±0.02 | 2.88±0.30 [31.1] | 0.96±0.13 | 5.16±0.30 [5.35] |
| FD-4 | 0.04±0.01 | 0.57±0.05 [13.3] | N.D. | 14.1±4.94 |

[ ]: enhancement ratio *versus* the control. N.D.: not detected. Each value represents mean±S.E. (*n*=4).

Table 3. Thickness of Corneal Epithelium and Stroma Following Application of Various Compounds

| | Control (BRS) | PLA | STDHF | LPS |
|---|---|---|---|---|
| Epithelial thickness ($\mu$m) | 24.8±0.61 | 27.9±1.16 | 18.2±1.40* | 15.2±1.43* |
| Stromal thickness ($\mu$m) | 175±3 | 182±3 | 184±6 | 362±19* |

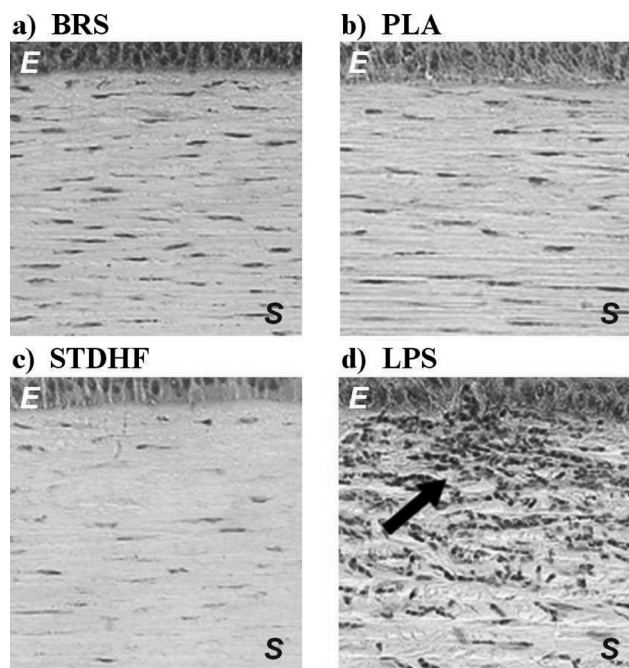*$p<0.01$ *vs.* control (Dunnet test). Each value represents mean±S.E. (*n*=4).

Fig. 2.　Cross-Section of the Cornea after Treatment with Various Compounds

(a) BRS (control), (b) PLA, (c) STDHF, (d) LPS, original magnification ×400. E; corneal epithelium, S; corneal stroma. Arrow shows the neutrophils infiltrated.
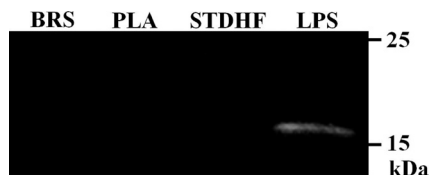


Fig. 3.　Production of TNF-$\alpha$ in Cornea after Repeated Instillation of Various Compounds

flunarizine,[17] brimonidine,[18] and timolol.[19]

In the presence of PLA at 5 mg/ml, pyridoxamine and FD-4 concentrations at 30 min after single instillation were increased 29- and 16-fold, respectively (Fig. 1), as well as the $AUC_{0-60}$ 28-fold for pyridoxamine and 18-fold for FD-4 compared with controls (Table 1). Podder et al.[20] reported that the timolol concentration in the aqueous humor achieved in the presence of 0.025% benzalkonium chloride and 0.05% EDTA after instillation of 0.65% timolol solution was 1.8- and 1.6-times higher than in the controls. It has also been reported that 0.025—1% laurocapram (Azone®) enhanced the area of under the aqueous humor concentration–time curve ($AUC_{\text{aqueous humor}}$) of cimetidine 3.9- to 2.6-fold using eye drops containing 1% cimetidine, and 0.025—1% hexamethylene lauramide increased the $AUC_{\text{aqueous humor}}$ by 4.4- to 19-fold.[21] The $AUC_{\text{aqueous humor}}$ following instillation of 0.2% ofloxacin solution containing 1% chitosan and N-carboxymethylchitosan as cationic polymers was 2.6- and 2.4-fold higher than that without cationic polymers (62.3 min· mg/ml), respectively.[22] Although Wang et al.[23] reported that cationized gelatin with ethylenediamine enhanced the absorption of FD-4 after nasal administration, its enhancing potency were lower than that of PLA,[24] suggesting that amount of positive charge in a molecule may have a significance for

the enhancing potency of polycations because there is a significant difference in number of amine residue between PLA and ethylenediaminated gelatin with comparable molecular weight. Thus, PLA appears to have the ability to increase drug absorption into the aqueous humor in a manner similar to that of those enhancers.

Polycationic enhancers including PLA may be useful for ocular drug delivery, since drug delivery into the vitreous body is extremely difficult because of the poor permeability of the surface of ocular tissues. Repeated instillation of PLA clearly increased the concentration of both pyridoxamine and FD-4 in aqueous humor and pyridoxamine in vitreous body compared with that in the control eye (Table 2). In addition, the appearance of FD-4 in the vitreous body after repeated instillation with PLA suggests that PLA has an ability to increase drug absorption into the vitreous body, even although the molecular size of drug is increased. Recently, the anti-vascular endothelial growth factor (VEGF) aptamer and the anti-VEGF antibody fragment were reported to be useful for the treatment of macular edema. These compounds are administrated by intravitreal injection.[25,26] However, direct injection of drugs into the vitreous body is stressful for patients and could potentially produce severe adverse events, such as endophthalmitis, retinal detachment, iritis/uveitis, ocular hypertension, cataract, intraocular hemorrhage, and hypotony.[27] Thus, non-invasive methods would be more acceptable to patients if a sufficient amount of drug could be delivered to the posterior segment of the eye.

In the case of drug delivery to the posterior segment, Williams et al.[28] showed that repetitive instillation of 0.5% sodium caprate in every 20 min over 12 h successfully increased the concentration of 28 kDa anti-rat CD4 antibody fragment (scFv) in vitreous body by 50—150 ng/ml while maintaining the scFv activity. Because caprate is known to increase the permeability of $\beta$-blockers (atenolol, carteolol, tilisolol, timolol, befunolol) 1.1- to 20.3-fold and 0.4- to 1.4-fold across the excised cornea and conjunctiva, respectively, in the separate studies,[29] this means that these absorption enhancers are useful for drug delivery to the posterior segment of eye, especially the delivery of protein preparations, such as the anti-vascular endothelial growth factor antibody fragment (ranibizumab)[30] approved for the treatment of age-related macular degeneration. Considering that capric acid increases the permeability of $\beta$-blockers (atenolol, carteolol, tilisolol, timolol, befunolol) by 1.1- to 20.3-fold and 0.4- to 1.4-fold in excised cornea and conjunctiva, respectively,[29] it was expected that PLA might have the same effect as sodium caprate on the delivery of protein preparations because PLA enhances the permeation of FD-4 and pyridoxamine by 6.8- to 8.0-fold in the cornea and 4.7- to 9.8-fold in the conjunctiva, respectively.[7]

PLA did not appear to induce significant inflammatory responses in the cornea as indicated by no change in the epithelial and stromal thickness of the cornea (Table 3), no infiltration of polymorphonuclear neutrophils (Fig. 2), and no production of inflammatory cytokine, TNF-$\alpha$ (Fig. 3). In addition, we demonstrated in a previous study that 0.5% PLA (50) did not affect the cellular viability of excised rabbit corneal and conjunctival epithelia.[7] The safety of PLA used as an absorption enhancer was also confirmed in the rabbit nasal epithelium as shown by histological observations and

no leakage of cellular components.[5,6] Thus, PLA is promising as a potential non-invasive absorption enhancer for transmucosal drug delivery as well as ocular delivery. Also, STDHF did not induce inflammatory responses as shown by the microscopic observations (Fig. 2c) and there was no production of TNF-$\alpha$ (Fig. 3). However, the significant reduction in the epithelial thickness of the cornea (Table 3) suggests possible cytotoxic effects in the rabbit cornea.[7] Bile salt derivatives like STDHF are known to produce detachment of epithelial cells in the rabbit nasal epithelium.[5,6]

In conclusion, PLA appears to enhance the absorption of FD-4 and pyridoxamine into intraocular tissues, such as aqueous humor and vitreous body without inducing any significant corneal damage and inflammation as far as the corneal thickness and TNF-$\alpha$ production are concerned. Consequently, PLA may be a useful absorption enhancer for ocular drug delivery. Investigations to identify ideal absorption enhancers may increase the potential use of instillation as an alternative to invasive intravitreal injection.

## REFERENCES

1) Mitra A. K., "Ophthalmic Drug Delivery System," Marcel Dekker, Inc., New York, 2003.
2) Thanou M., Verhoef J. C., Junginger H. E., *Adv. Drug Deliv. Rev.*, **50** (Suppl. 1), S91—S101 (2001).
3) van der Lubben I. M., Verhoef J. C., Borchard G., Junginger H. E., *Eur. J. Pharm. Sci.*, **14**, 201—207 (2001).
4) Wang J., Sakai S., Deguchi Y., Bi D., Tabata Y., Morimoto K., *J. Pharm. Pharmacol.*, **54**, 181—188 (2002).
5) Natsume H., Iwata S., Ohtake K., Miyamoto M., Yamaguchi M., Hosoya K., Kobayashi D., Sugibayashi K., Morimoto Y., *Int. J. Pharm.*, **185**, 1—12 (1999).
6) Ohtake K., Natsume H., Ueda H., Morimoto Y., *J. Control Release*, **82**, 263—275 (2002).
7) Nemoto E., Takahashi H., Kobayashi D., Ueda H., Morimoto Y., *Biol. Pham. Bull.*, **29**, 155—160 (2006).
8) Ohtake K., Maeno T., Ueda H., Natsume H., Morimoto Y., *Pharm. Res.*, **20**, 153—160 (2003).
9) Ohtake K., Maeno T., Ueda H., Ogihara M., Natsume H., Morimoto Y., *Pharm. Res.*, **20**, 1838—1845 (2003).
10) Curren R. D., Harbell J. W., *Altern. Lab. Anim.*, **30** (Suppl.), 69—74 (2002).
11) Wilhelmus K. R., *Surv. Ophthalmol.*, **45**, 493—515 (2001).
12) Bisp M., Bor M. V., Heinsvig E. M., Kall M. A., Nexo E., *Anal. Biochem.*, **305**, 82—89 (2002).
13) Khatri S., Lass J. H., Heinzel F. P., Petroll W. M., Gomez J., Diaconu E., Kalsow C. M., Pearlman E., *Invest. Ophthalmol. Vis. Sci.*, **43**, 2278—2284 (2002).
14) Holzer M. P., Solomon K. D., Vroman D. T., Vargas L. G., Sandoval H. P., Kasper T. J., Apple D. J., *J. Cataract Refract. Surg.*, **29**, 542—549 (2003).
15) Laemini U. K., *Nature* (London), **227**, 680—685 (1970).
16) Sakanaka K., Kawazu K., Tomonari M., Kitahara T., Nakashima M., Kawakami S., Nishida K., Nakamura J., Sasaki H., *Pharm. Res.*, **21**, 770—776 (2004).
17) Maltese A., Bucolo C., *J. Ocul. Pharmacol. Ther.*, **19**, 171—179 (2003).
18) Acheampong A. A., Shackleton M., John B., Burke J., Wheeler L., Tang-Liu D., *Drug Metab. Dispos.*, **30**, 421—429 (2002).
19) Yamamura K., Sasaki H., Nakashima M., Ichikawa M., Mukai T., Nishida K., Nakamura J., *Pharm. Res.*, **16**, 1596—1601 (1999).
20) Podder S. K., Moy K. C., Lee V. H., *Exp. Eye Res.*, **54**, 747—757 (1992).
21) Ismail I. M., Chen C. C., Richman J. B., Andersen J. S., Tang-Liu D. D., *Pharm. Res.*, **9**, 817—821 (1992).
22) Di Colo G., Zambito Y., Burgalassi S., Nardini I., Saettone M. F., *Int. J. Pharm.*, **273**, 37—44 (2004).
23) Wang J., Sakai S., Deguchi Y., Bi D., Tabata Y., Morimoto K., *J. Pharm. Pharmacol.*, **54**, 181—188 (2002).
24) Miyamoto M., Natsume H., Iwata S., Ohtake K., Yamaguchi M., Kobayashi D., Sugibayashi K., Yamashina M., Morimoto Y., *Eur. J. Pharm. Biopharm.*, **52**, 21—30 (2001).
25) Gragoudas E. S., Adamis A. P., Cunningham E. T., Jr., Feinsod M., Guyer D. R., *N. Engl. J. Med.*, **351**, 2805—2816 (2004).
26) Cunningham E. T., Jr., Adamis A. P., Altaweel M., Aiello L. P., Bressler N. M., D'Amico D. J., Goldbaum M., Guyer D. R., Katz B., Patel M., Schwartz S. D., *Ophthalmology*, **112**, 1747—1757 (2005).
27) Jager R. D., Aiello L. P., Patel S. C., Cunningham E. T., Jr., *Retina*, **24**, 676—698 (2004).
28) Williams K. A., Brereton H. M., Farrall A., Standfield S. D., Taylor S. D., Kirk L. A., Coster D. J., *Eye*, **19**, 910—913 (2005).
29) Sasaki H., Igarashi Y., Nagano T., Nishida K., Nakamura J., *Pharm. Res.*, **12**, 1146—1150 (1995).
30) Steinbrook R., *N. Engl. J. Med.*, **533**, 1409—1412 (2006).