Development of Latanoprost-Loaded Biodegradable Nanosheet as a New Drug Delivery System for Glaucoma

Kenji Kashiwagi,1 Keisuke Ito,2 Hiroki Haniuda,2 Shinya Ohtsubo,3 and Shinji Takeoka2

1Department of Ophthalmology, University of Yamanashi Faculty of Medicine, Chuo, Yamanashi, Japan
2Department of Life Science and Medical Bioscience, Graduate School of Advanced Science and Engineering, Waseda University, Shinjuku, Tokyo, Japan
3Consolidated Research Institute for Advanced Science and Medical Care, Waseda University, Shinjuku, Tokyo, Japan

Glucoma is a disease that causes irreversible visual function loss and a majority of glaucoma patients require lifelong care. So far, IOP reduction is the only proven therapy. Most patients use antiglaucoma ophthalmic solutions and it is estimated that each patient uses an average of two antiglaucoma ophthalmic solutions.1 However, studies have revealed that glaucoma patients show poor adherence to antiglaucoma ophthalmic solutions2 and adherence worsens with an increase in the number of antiglaucoma ophthalmic solutions used.3 Okeke et al. reported that ophthalmologists could not monitor adherence accurately.4 In addition to the poor adherence and difficulty of monitoring adherence, unnecessary administration into the cul-de-sac is another important problem of topical antiglaucoma therapy. It has been reported that the topical administration of ophthalmic solutions has lower continuity than the oral administration of drugs.5 Stone et al. demonstrated that patients with ocular hypertension and glaucoma showed poor adherence to topical therapy with ocular hypotensive agents.6 Dietlein et al. reported that adherence to therapy with antiglaucoma ophthalmic solutions deteriorated with age.7

Several novel drug delivery systems (DDSs) aimed at addressing the issue of adherence and ensuring consistent IOP reduction are under development. Various systems have been proposed, including contact lenses,8–11 ocular inserts,12,13 and injectable biodegradable micro- and nanoparticles.14–17 The emergence of nanomaterials and nanometer-order devices that are applicable to ophthalmology is highly awaited.18 We have developed a novel layer-by-layer method that uses sodium alginate as the polyanion and chitosan as the polycation to create a “nanosheet,” a biodegradable and biocompatible film of nanometer thickness.19,20 We also have developed a novel biodegradable nanosheet that carries drugs, such as tetracycline, as a new antibiotic DDS.21 The nanosheet features interesting properties as a result of its nanometer thickness, such as high flexibility, high adhesiveness even without any adhesive agents, and high transparency. It is expected that adverse effects would be minimized by applying only a very small amount of biodegradable polymer to the ocular surface. Taken together, a biodegradable nanosheet containing an antiglaucoma ophthalmic drug may be a good candidate for a new DDS for glaucoma.

In our study, we prepared a latanoprost-loaded biodegradable nanosheet (LBNS), because latanoprost is a highly popular antiglaucoma ophthalmic drug. We investigated the magnitude and duration of IOP reduction, and the safety of LBNS.

Correspondence: Kenji Kashiwagi, Department of Ophthalmology, University of Yamanashi Faculty of Medicine, Chuo, Yamanashi, Japan; kenji@yamanashi.ac.jp.

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PURPOSE. We investigated the IOP reduction and safety of latanoprost-loaded biodegradable nanosheet (LBNS) as a new antiglaucoma drug delivery system (DDS).

METHODS. We fabricated a 40 nm thick multilayered biodegradable nanosheet that is composed of chitosan and sodium alginate by means of the layer-by-layer method. Latanoprost isopropyl ester was loaded on the nanosheet to prepare 25, 2.5, and 0.25 μg/cm² LBNSs. A nanosheet without latanoprost isopropyl ester (NS) and 0.005% latanoprost ophthalmic solution were prepared as controls. LBNSs or NS was applied to rat cornea, and IOP was monitored for 9 days. Local adverse effects and eye scratching movement also were investigated. The amount of latanoprost acid in aqueous humor and was measured in rabbits.

RESULTS. The 0.25 μg/cm² LBNS and 0.005% latanoprost ophthalmic solution showed significant IOP reduction only for 1 day after application, whereas the IOP reduction rates of 2.5 μg/cm² LBNS at 1, 2, 4, 7, and 9 days after application were −27.0% ± 14.8%, −22.0% ± 16.7%, −25.8% ± 18.0%, −22.7% ± 20.9%, and −6.6% ± 17.0%, respectively. The 25 μg/cm² LBNS reduced IOP in a similar manner. The 25 μg/cm² LBNS induced transient hyperemia, whereas the 0.25 and 2.5 μg/cm² LBNSs did not exert any local adverse effects. The eye scratching movement test showed that application of 25 μg/cm² LBNS did not cause any irritation of the eye. Latanoprost acid was detected in aqueous humor up to 6 days after application of 2.5 μg/cm² LBNS.

CONCLUSIONS. LBNS may be used as a novel antiglaucoma DDS.

Keywords: drug delivery system, latanoprost, chitosan, sodium alginate, glaucoma, nanosheet
**Materials and Methods**

All experiments were conducted in accordance with the ARVO Animal Statement for the Use of Animals in Ophthalmic and Vison Research.

**Preparation of LBNS**

Chitosan (molecular weight [MW] = 88,000) and sodium alginate (MW = 106,000) were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Polyvinyl alcohol (PVA, MW = 22,000) was purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). Those polymers were used as purchased.

All procedures for LBNS fabrication were conducted in a class 10,000 clean room to avoid contamination. Chitosan (1 mg/mL, 1% [vol/vol] acetic acid) and sodium alginate (1 mg/mL) solutions were prepared by using deionized water (DI). The spin-coating-assisted layer-by-layer method was performed as described in previous reports with a spin coater (Opticoat MS-A150; Mikasa Co. Ltd., Tokyo, Japan). The scheme for LBNS preparation is summarized in Figure 1.

Briefly, 1 mL of chitosan solution was deposited on a SiO$_2$ substrate by dropping and the substrate was spin-coated at 4500 revolutions per minute (rpm) for 15 seconds. The substrate was rinsed twice with DI while being spun at 4500 rpm for 15 seconds. Then, 1 mL of sodium alginate solution was spin-coated and the substrate was rinsed with DI using the same conditions as those for chitosan. This procedure was repeated 10 times, terminating at the chitosan spin-coating step. Next, 10 wt% PVA solution was cast on the surface of the fabricated nanosheet and the resulting composite film was peeled off the substrate with tweezers after drying in vacuo. The film was turned upside down and deposited onto another SiO$_2$ substrate with the PVA side facing the substrate surface. Next, latanoprost isopropyl ester solution was cast on the surface of the chitosan/alginate multilayered nanosheet and the nanosheet was dried in vacuo. We also prepared a chitosan/alginate multilayered nanosheet without latanoprost isopropyl ester as control. In this study, we prepared a series of LBNSs with the following amounts of latanoprost loaded: 25, 2.5, and 0.25 μg/cm$^2$, as determined from assumptions and data of preliminary studies described as follows: (1) LBNS measuring 2 × 2 mm in size is to be applied to the corneal surface, (2) the percentage reduction of LBNS due to the release of loaded latanoprost is approximately 2.5%, (3) one drop of 0.005% latanoprost ophthalmic solution (5 μL) contains approximately 0.25 μg of latanoprost, and (4) less than 5% of instilled ophthalmic solution penetrates into the anterior chamber. The amounts of latanoprost in 25, 2.5, and 0.25 μg/cm$^2$ LBNSs were estimated to be 1.0, 0.1, and 0.01 μg, respectively.

**Procedure for Applying LBNS to Corneal Surface**

Adult Sprague-Dawley (SD) rats weighing 150 to 200 g were used. The rats were anesthetized with an abdominal injection of pentobarbitone. After the tears on the ocular surface were absorbed with a piece of absorbable paper, LBNS measuring 2 × 2 mm in size was applied gently to the center of the cornea of a randomly selected eye with the latanoprost side on the ocular surface, with a surgical microscope (OPMI 6SH; Carl Zeiss Meditec, Jena, Germany). Then, blinking was induced several times. A nanosheet without latanoprost of the same size was applied on the other eye as control in the same fashion.

**Magnitude and Duration of IOP Reduction by LBNS**

After general anesthesia as described above, IOP was measured with a rebound tonometer (TonoLab; Icare Finland, Espoo, Finland). As TonoLab had provided a reliability score of 5% or less as the reliable values according to the manufacturer’s recommendation, IOP measurements were repeated until three reliable measurements could be obtained and the mean of the three measurements was used as the IOP value for that set of measurements. IOP measurements were performed immediately before applying LBNS or control to the corneal surface, and at 1, 2, 4, 7, and 9 days after application.

One drop of 0.005% latanoprost ophthalmic solution (approximately 5 μL) was instilled on a randomly selected rat eye and one drop of saline (approximately 5 μL) was instilled on another eye as control. In one group, 0.005% latanoprost ophthalmic solution was instilled only once, and IOP measurements were performed immediately before instilling 0.005% latanoprost ophthalmic solution or saline on the corneal surface, and at 1, 2, 4, and 7 days after the instillation. In another group, 0.005% latanoprost ophthalmic solution or saline was instilled once a day for four days and IOP measurements were performed immediately before instilling 0.005% latanoprost ophthalmic solution or saline on the corneal surface; and at 1, 2, 4, and 7 days after the first instillation.

**Concentrations of Latanoprost in Aqueous Humor**

To investigate latanoprost transport into aqueous humor, we used adult Japanese white rabbits weighing 1.5 to 1.8 kg, because the amount of aqueous humor from rat eye was not sufficient for measurement. The rabbits were anesthetized with an intravenous injection of pentobarbitone. Then, 25 or 2.5 μg/cm$^2$ LBNS measuring 6 × 6 mm in size was applied to the center of the cornea of the right eye after wiping off ocular surface tears with the same procedure as that applied to rats. The size of LBNS for the rabbit eye was determined by assuming that the amount of aqueous humor in rabbits was approximately 10-fold larger than that in rats. No procedure was performed on the left eye. A 30-gauge needle was inserted into the anterior chamber and aqueous humor samples were collected carefully, avoiding contamination with tears on the ocular surface. Approximately 0.2 mL of aqueous humor sample was aspirated from the limbus at 6 hours, and 1, 3, 5, 6, and 7 days after LBNS application. Samples from the left eyes were collected as control. Approximately 40 μL of 0.005% latanoprost ophthalmic solution or saline was instilled on a randomly selected rabbit eye. Using a 30-gauge needle, 0.2 mL of aqueous humor was aspirated from the limbus at 2 hours, and 1 and 2 days after instillation. Samples were stored at −80°C until measurement.

**Pharmacokinetics of Latanoprost From LBNS In Vitro**

The release of latanoprost from LBNS was investigated in vitro. One 2.5 μg/cm$^2$ LBNS measuring 2 × 2 cm was placed on the bottom of a plastic microplate (nontreated MICROPLATE, code 1810-006; Iwaki Co. Ltd., Tokyo, Japan). All edges of the LBNS were sealed by carbon tape to prevent latanoprost release from the space between the microplate bottom and the LBNS. Then, 5 mL of saline were poured into the plastic microplate. Saline containing released latanoprost was recovered, and another 5 mL of saline was poured again into the plastic microplate to analyze the amount of latanoprost in saline at 0.5, 1, 2, 3, 6, and 25 hours after incubation at room temperature. The samples were stored at −80°C until measurement. We prepared LBNSs consisting of 5, 10, and 20 chitosan-sodium alginate bilayers for this purpose. Four conditions were set to investigate the kinetics of latanoprost release: (1) A 2.5 μg/
cm² LBNS consisting of 10 chitosan-sodium alginate bilayers was placed on the microplate with the latanoprost-containing layer facing up. (2) A 2.5 µg/cm² LBNS consisting of five chitosan-sodium alginate bilayers was placed on the microplate with the latanoprost-containing layer facing down. (3) A 2.5 µg/cm² LBNS consisting of 10 chitosan-sodium alginate bilayers was placed on the plate with the latanoprost-containing layer facing down. And (4) A 2.5 µg/cm² LBNS consisting of 20 chitosan-sodium alginate bilayers was placed on the plate with the latanoprost-containing layer facing down.

Then, we investigated the accuracy of the amount of latanoprost loaded in LBNS. One 2.5 µg/cm² LBNS measuring 2 × 2 cm was placed at the bottom of the plastic microplate and 5 mL of saline were poured into the plastic microplate as indicated above. Saline containing released latanoprost was recovered and another 5 mL of saline was poured again into the plastic microplate periodically up to 120 hours. The recovered saline solutions were analyzed for the amount of latanoprost. This procedure was repeated until latanoprost could not be detected in the saline samples. The total amount of latanoprost loaded in LBNS was expressed as the sum of the weights of latanoprost in the saline samples.

Measurement of Latanoprost

A commercially available enzyme immunoassay (EIA) kit (Catalog #516811; Cayman Chemical Company, Ann Arbor, MI) was used to measure the amount of latanoprost. This kit can measure latanoprost isopropyl ester and latanoprost acid. The amounts of latanoprost acid were measured in aqueous humor samples, because latanoprost isopropyl ester was converted into latanoprost acid, the biologically active form, as it passed into the anterior chamber. Then, we prepared a latanoprost acid standard curve. As a preliminary experiment, we diluted samples with several dilution ratios from 100X to 50X to determine the most appropriate dilution ratio. Aqueous humor was diluted with an adequate dilution ratio with EIA buffer and 50 µL of the diluted sample was added directly to each assay well that was precoated with antibody. Incubation was done for 2 hours at room temperature on an orbital shaker. The sample solutions were dumped and the wells were rinsed five times with washing buffer. Then, 200 µL of Ellman’s reagent were added into each well. The assay wells were shaken for 60 minutes at room temperature. The plate was read at the wavelength of 412 nm using a plate reader (SpectraMax Plus 384; Molecular Devices, Sunnyvale, CA). The concentrations of latanoprost were determined from the standard curve. The limit of detection of EIA was 3.9 nM.

Examination of Local Adverse Effects of LBNS

We examined the local adverse effects of LBNS on adult SD rats. Eyes to which 25, 2.5, or 0.25 µg/cm² LBNS was applied and control eyes were subjected to examination with a surgical microscope (OPMI 6SH) and a handheld slit-lamp microscope (Kowa, SL-15, Kowa, Nagoya, Japan) before application and at 6 hours, and 1, 2, 3, 5, 7, and 9 days after application. Eyes to which 25 µg/cm² LBNS were applied were subjected to histologic examination. Eyes were enucleated at 1 and 7 days after LBNS application, fixed in 4% paraformaldehyde, deparaffinized in xylene, hydrated in graded ethanol, stained with hematoxylin and eosin, and mounted for observation.

Evaluation of Eye Scratching Movement After LBNS Application

Adult SD rats were used in this experiment. To one randomly selected eye was applied 25 µg/cm² LBNS or control and a sham procedure was performed on the other eye as control. Each rat was housed solely in an observation cage and the frequency of eye scratching movement was recorded by a video camera (Canon HFM32; Canon, Tokyo, Japan) twice for 30 minutes each at 6 and 8 hours after application, and the mean number of eye scratching movements was subjected to analysis. The number of eye scratching movements was counted according to Nakano’s protocol. In brief, eye scratching movement was defined by an uninterrupted cluster of rapid fore- or hind limb movements directed to the ocular surface.

Statistical Analysis

All data are expressed as means ± SD. Statistical analysis was performed using one-way ANOVA followed by Dunnett’s test for multiple comparisons. Student’s t-test or the Mann-Whitney U test was used for comparison between two groups. P < 0.05 was considered significant.

RESULTS

Although LBNS was very thin and had high transparency, it was easy to handle under the surgical microscope. After applying

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Scheme for preparation of latanoprost-loaded biodegradable nanosheet.
one edge of LBNS to the corneal surface, the LBNS was easily spread out and firmly adhered to the corneal surface. Although some wrinkles were observed in the LBNS immediately after application to the corneal surface, the approximately 10 µm thick PVA layer dissolved soon after blinking several times and the LBNS could not be identified even by slit-lamp examination (Fig. 2). Please refer to Supplementary Movie S1 for the handling of LBNS and its application to the corneal surface.

Magnitude and Duration of IOP Reduction by LBNS

Figure 3 shows an IOP reduction profile after 2.5 µg/cm² LBNS were applied to the corneal surface. LBNS at 2.5 µg/cm² significantly reduced IOP at 1, 2, 4, and 7 days after application compared to the control. The IOP reduction rate was calculated as the percentage difference in IOP reduction between LBNS eyes and control eyes at each measurement point. The IOP reduction rates of LBNS at 1, 2, 4, 7, and 9 days after application were −27.0% ± 14.8%, −22.0% ± 16.7%, −25.8% ± 18.0%, −22.7% ± 20.9%, and −6.6% ± 17.0%, respectively. There were significant differences in the IOP reduction rate between LBNS eyes and control eyes at 1, 2, 4, and 7 days after application. The IOP reduction rates at 1, 2, 4, and 7 days after application of 2.5 µg/cm² LBNS were not significantly different.

In contrast, a single dose of 0.005% latanoprost ophthalmic solution significantly reduced IOP only at 1 day after application. Meanwhile, IOP of eyes instilled with 0.005% latanoprost ophthalmic solution once a day for four consecutive days was significantly reduced at 1, 3, and 4 days after instillation from the baseline, but returned to the baseline by 7 days after the first instillation (Fig. 4).

Dependence of Magnitude and Duration of IOP Reduction on Latanoprost Dose (Fig. 5)

The three different doses of LBNS (0.25, 2.5, and 25 µg/cm²) reduced IOP significantly at 1 day after application and the maximum IOP reduction was not significantly different among them, although a higher concentration tended to show greater IOP reduction. Of note was that 0.25 µg/cm² LBNS did not show significant IOP reduction at 2 days after application and later. Both 25 µg/cm² and 2.5 µg/cm² LBNSs showed significant IOP reduction that lasted longer than with 0.25 µg/cm² LBNS. There was no significant difference in IOP reduction in eyes treated with 25 and 2.5 µg/cm² LBNSs throughout the experiment.

Pharmacokinetics of Latanoprost From LBNS In Vitro

Figure 6 compares the release kinetics for three conditions. A 2.5 µg/cm² LBNS placed on the microplate with the latanoprost-containing layer facing up released more than 80% of the loaded latanoprost within 30 minutes and the loaded latanoprost was released almost completely in 2 hours. In contrast, the release of latanoprost from 2.5 µg/cm² LBNS was prolonged when the LBNS was placed on the microplate with the latanoprost-containing layer facing down. LBNS having a larger number of chitosan-sodium alginate bilayers showed a much slower release of latanoprost.

Expected amount of latanoprost in 2.5 µg/cm² LBNS measuring 2 × 2 cm was 10 µg, and the total amount of latanoprost released from the 2 × 2 cm 2.5 µg/cm² LBNS was 9.95 ± 0.49 µg (N = 5).

Figure 2. Appearance and application of LBNS to the corneal surface. (a) LBNS was picked up from a culture dish with micro forceps. (b) LBNS was applied gently to the corneal surface. (c) Wrinkles of LBNS were observed immediately after application. (d) Contour of LBNS was almost undetectable after blinking several times.

Figure 3. Profile of IOP reduction by LBNS. *P < 0.05, Mann-Whitney U test, N = 6 to 12, bar: SD.
Concentration of Latanoprost Acid in Aqueous Humor

The concentrations of latanoprost acid in the aqueous humor of rabbits are depicted in Figure 7. Latanoprost acid was detected at 6 hours, and 1, 3, 5, and 6 days after LBNS application. The 25 μg/cm² LBNS induced the production of a significantly higher concentration of latanoprost acid than the 2.5 μg/cm² LBNS at 6 hours after application, and no significant difference between the two concentrations was noted at other measured points. Latanoprost acid was detected only at 2 hours after instillation of 0.005% latanoprost ophthalmic solution. No latanoprost acid was detected in the aqueous humor from rabbit eyes without LBNS application or from eyes instilled with saline.

Adverse Effects

Slit-lamp examination revealed hyperemia in rats treated with 25 μg/cm² LBNS only at 6 hours after application; nevertheless, this condition was transient. Hyperemia disappeared at 1 day after application and other local adverse effects, including hypophagia, corneal erosion, corneal edema, inflammatory signs in the anterior chamber, and lens opacity, were not observed throughout the experiment. LBNS at 2.5 and 0.25 μg/cm² showed no signs of local adverse effects at any examination points.

Histologic Examination

Figure 8 shows the results of histologic examination of the cornea and retina at 1 or 7 days after 25 μg/cm² LBNS application. Even when LBNS with the highest latanoprost concentration was applied, no adverse effects, such as cell infiltration, edema, and hemorrhage, in conjunctiva, cornea, iris, lens, vitreous cavity, and fundus were observed.

Eye Scratching Movement

There was no significant difference in rat eye scratching movement of the hind limbs and forelimbs between control eyes and eyes to which LBNS was applied (Fig. 9a). No
significant difference in rat eye scratching movement also was observed between control eyes and eyes that were subjected to the sham procedure (Fig. 9b).

**DISCUSSION**

Our study revealed that a single application of LBNS to the corneal surface significantly reduced IOP for approximately one week without producing any severe adverse effects and that the extent of IOP reduction was almost the same as that of 0.005% latanoprost ophthalmic solution.

Problems encountered in topical glaucoma therapy include poor adherence and inadequate administration. Glaucoma patients tend to be free of symptoms until severe damage in the optic nerve becomes apparent, and they cannot recognize the efficacy of antiglaucoma ophthalmic solutions by themselves. The incidence of glaucoma increases among the elderly who find it difficult to instill eye drops properly. Under the current circumstances that many glaucoma patients require lifelong therapy, we are required to develop a new paradigm that would alleviate patient’s burden and support lifelong care of glaucoma. As LBNS relieves patients of the task of daily instillation of antiglaucoma ophthalmic solutions, it may be one of the options for glaucoma therapy.

In ophthalmology as well as other medical fields, the development of DDSs using nanotechnology is being promoted. Vesicles, micelles, emulsions, and biodegradable nanoparticles have been investigated as carriers for drugs. DDSs that use nanotechnology are able to release drugs over a longer period than currently available local antiglaucoma drugs; however, some DDSs require surgery, while others require carrier materials, such as contact lenses. Compared to previously reported DDSs, LBNS possesses the following characteristics.

This material is available as a thin film...
and its dimensions are very unique. As its thickness is of nanometer order, and its width and length are of centimeter order, self-standing is realized, unlike other nanomaterials. It is possible to control its dimensions to arbitrary length and width or even thickness if necessary. In our study, we used chitosan and sodium alginate. Chitosan is a cationic polymer that has favorable biological characteristics. Sodium alginate is an anionic polymer that is used widely in medicine. The multilayered nanosheet using polycations and polyanions allows the loading of lipophilic and hydrophilic compounds without the need for any chemical modifications. This material can be loaded with drugs other than latanoprost. Indeed, we have succeeded in loading this sheet with a beta-blocker ophthalmic solution (data not shown). We routinely performed quality check of fabricated LBNS containing a specific amount of latanoprost and confirmed that LBNS has good reproducibility. This material can relieve patients of the burden of having to apply antiglaucoma ophthalmic solutions daily, particularly patients who must instill several antiglaucoma drugs or have difficulty instilling antiglaucoma ophthalmic solutions.

LBNS is an adhesive preparation applicable to the cornea, and is similar to contact lens type DDSs. However, there are several differences between LBNS and contact lens type DDSs. The most important difference is the thickness of the material. The thickness of LBNS used in our study is approximately 40 nm, whereas that of contact lens type DDSs is approximately 500 μm at the center. We have reported that a nanometer-thick sheet shows an exponential increase in adhesiveness and high flexibility if its thickness is below approximately 250 nm. As LBNS adheres strongly to the corneal surface and a 4 μm tear layer covers it, LBNS is completely buried in the tear layer. Because of these differences, LBNS is almost free from conventional contact lens-related complications, such as foreign body sensation, chronic inflammation, and allergic reactions related to the materials used. As LBNS is very thin and its refractive index is almost the same as that of the cornea, LBNS may not affect refraction. LBNS is biodegradable and degraded in approximately 10 days in phosphate buffered saline (unpublished data). Thus, it is not necessary to remove the existing LBNS before applying a new LBNS.

Since the EIA kit used rabbit’s IgG antibody, there is a possibility that EIA may detect antibodies interfering with the measurement. We confirmed this as we measured the concentration of latanoprost in samples accurately without any pretreatment by the following two supplemental experiments. Firstly, we measured the amount of latanoprost in the eye that was not treated with latanoprost. This EIA kit did not detect latanoprost in that eye. Secondary, we added pre-measured latanoprost acid into aqueous humor from healthy rabbit’s eye. An amount of latanoprost acid in this mixture was measured using a completely same manner as in this study. Measured concentrations of latanoprost acid correlated well with calculated concentrations of latanoprost acid (please see Supplementary Fig. S1). We believe that the currently used EIA kit can measure the concentration of latanoprost correctly.

As the polyvinyl alcohol layer weighed much more than the loaded latanoprost, we estimated the amount of loaded latanoprost by determining the sum of the weights of latanoprost released in saline. We found that the current LBNS assembling system had good accuracy and reproducibility of latanoprost loading in the biodegradable nanosheet.

Pharmacokinetics studies revealed that the LBNS placed on the microplate with the latanoprost-containing layer facing down showed significantly prolonged release of latanoprost compared to that with the latanoprost-containing layer facing up. However, this in vitro experiment showed that approximately 80% of the loaded latanoprost in 2.5 μg/cm² LBNS was released in 24 hours when the latanoprost-containing layer was facing down, which seemed to contradict the current in vivo study that LBNS reduced IOP for seven days. Because it was impossible to compare directly the results of in vitro and in vivo experiments, further investigations should be conducted to clarify details of the pharmacokinetics. Our study also indicated the possibility of controlling drug release by varying the number of bilayers. It is necessary to investigate the optimal conditions for longer IOP reduction by LBNS than in our study.

Our study showed that latanoprost acid released from LBNS could be detected in aqueous humor up to 6 days after application, although the concentrations in aqueous humor at 1 day after application or later were below the pharmacologically effective concentration, and the level of latanoprost detected in the aqueous humor decreases significantly each day. Theoretically, latanoprost below the pharmacologically effective concentration may not reduce IOP. We offer the following explanation for this discrepancy. Latanoprost binds to the FP receptor of ciliary muscle cell to reduce IOP and, thus, its concentration in the aqueous humor does not always reflect that in the ciliary muscle cell. As we used rabbits in this study, we could not simply apply the results to the rat study. According to a drug information report, the concentration of latanoprost acid in the anterior chamber became undetectable within 1 day after instillation, which was confirmed in our study. The concentration of latanoprost acid in the aqueous humor was below the pharmacologically effective concentration at approximately 12 hours after instillation of conventional eye drops, although IOP reduction persisted for approximately 1 day after the instillation. Although our study showed that the longer latanoprost exists in the anterior chamber, the longer is the duration of IOP reduction, and further investigation is warranted.

The three concentrations of LBNS showed similar maximum IOP reduction, but the 0.25 μg/cm² LBNS showed shorter
duration of IOP reduction than the other two concentrations. The calculated amount of latanoprost in 0.25 \( \mu \text{g/cm}^2 \) LBNS measuring \( 2 \times 2 \text{ mm} \) in size was equivalent to 0.2 \( \mu \text{L} \) of 0.005\% latanoprost ophthalmic solution. Thus, LBNS at 0.25 \( \mu \text{g/cm}^2 \) is considered to satisfy the minimum amount of latanoprost required to induce maximum IOP reduction. However, that amount is not sufficient to sustain IOP reduction. LBNS at 25 \( \mu \text{g/cm}^2 \) and 2.5 \( \mu \text{g/cm}^2 \) showed similar potential for IOP reduction and LBNS at 25 \( \mu \text{g/cm}^2 \) produced local adverse effects for a short period after application. The initial amount of latanoprost released after the application of 25 \( \mu \text{g/cm}^2 \) LBNS might be larger than that after application of 2.5 \( \mu \text{g/cm}^2 \) LBNS, and this might have resulted in transient hyperemia. Taken together, LBNS at 2.5 \( \mu \text{g/cm}^2 \) is the most suitable for clinical use in this regard.

In this study, a relatively wide variation of IOP values was noted. It is known that IOP measurement with a rebound tonometer results in a relatively wide variation of IOP values. Differences in individual response to latanoprost may be another reason for the variation. IOP shows a relatively large day-to-day variation, while difference in IOP between the right and left eyes is relatively small. That is why we compared IOP between the experimented eyes and the control eyes in this study.

In vivo observation and pathologic examination showed no apparent adverse effects induced by LBNS, except transient hyperemia that resulted from the application of 25 \( \mu \text{g/cm}^2 \) LBNS. The eye scratching movement test also showed that LBNS did not irritate the eye. We did not investigate systemic adverse effects in our study. A pharmaceutical company released a safety report documenting that intravenous infusion of up to 3 \( \mu \text{g/kg} \) in healthy volunteers produced mean plasma concentrations that were 200 times higher than that during clinical treatment, yet resulted in no systemic adverse effects. The amounts of latanoprost in rat eyes treated with 25 \( \mu \text{g/cm}^2 \) LBNS and 2.5 \( \mu \text{g/cm}^2 \) LBNS were approximately 5 \( \mu \text{g/kg} \) (1.0 \( \mu \text{g}/200 \text{ g} \)) and 0.5 \( \mu \text{g/kg} \) (0.1 \( \mu \text{g}/200 \text{ g} \)), respectively.

Glaucoma is a common ocular disease. Approximately 60 million people are affected worldwide and 8.4 million are bilaterally blind.\(^{27}\) DDSs should be safe, easy to apply, and effective. Some DDSs require surgery,\(^{17}\) while others cause discomfort in patients. Unfortunately, there is no clinically available DDS whose efficacy lasts for more than one day. Our study showed the potential of LBNS as a new DDS for glaucoma therapy. However, the following issues should be resolved before LBNS can be used clinically. First, the efficacy of a single dose lasts for approximately 7 days, which means patients must apply LBNS every week. LBNS is 40 nm thick and is degraded in approximately 10 days. Thus, it is possible to apply a new LBNS on the initially applied LBNS if further IOP reduction is required. In this regard, a DDS with much longer efficacy is necessary. Second, handling of the current LBNS requires technical training and, thus, it may be difficult for patients to apply LBNS by themselves, although it is not difficult for medical staff to handle LBNS as part of routine patient care at outpatient clinics. Thus, an improvement of LBNS handling is necessary. Third, we used rats in the current study. Although Aihara et al. reported that rats showed IOP reduction similar to that in humans,\(^{29}\) we must conduct studies using monkey or other primates before a human study.

In conclusion, a single application of LBNS reduced IOP for approximately one week without any severe adverse effects. In this regard, LBNS shows potential as a new DDS for glaucoma therapy and is expected to be useful for glaucoma patients, especially those with poor adherence, and those who encounter difficulties in daily administration.

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