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Research Article

Cationic Gelatin Nanoparticles for Drug Delivery to the Ocular Surface: *In Vitro* and *In Vivo* Evaluation

Ching-Li Tseng,[1] Ko-Hua Chen,[2,3,4] Wen-Yu Su,[2,5,6] Yen-Hsien Lee,[2,7] Chi-Chang Wu,[1] and Fen-Huei Lin[2,5]

[1] *Graduate Institute of Biomedical Materials and Tissue Engineering, College of Oral Medicine, Taipei Medical University, No. 250, Wu-Hsing Street, Taipei City 110, Taiwan*
[2] *Division of Medical Engineering Research, National Health Research Institutes, No. 35, Keyan Road, Zhunan Town, Miaoli County 350, Taiwan*
[3] *Department of Ophthalmology, Taipei Veterans General Hospital, No. 201, Section 2, Shipai Road, Beitou District, Taipei City 112, Taiwan*
[4] *National Yang-Ming University, No. 155, Section 2, Linong Street, Taipei City 112, Taiwan*
[5] *Institute of Biomedical Engineering, National Taiwan University, No. 1, Section 1, Ren-ai Road, Taipei City 100, Taiwan*
[6] *Institute of Biomedical Engineering and Material Science, Central Taiwan University of Science and Technology, No. 666, Buzih Road, Taichung City 406, Taiwan*
[7] *Graduate Institute of Medical Science, College of Medicine, Taipei Medical University, No. 250, Wu-Hsing Street, Taipei City 110, Taiwan*

Correspondence should be addressed to Fen-Huei Lin; double@ntu.edu.tw

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To develop an effective ocular drug delivery carrier, we prepared two different charged gelatin nanoparticles (GPs) and evaluated particle size, surface charge, and morphology. The *in vitro* biocompatibility of GPs was assessed using human corneal epithelium (HCE) cells and *in vivo* safety by administering them as eye drops to New Zealand rabbits. The GPs prepared using type A gelatin were positively charged (GP(+), +33 mV; size, 180.6 ± 45.7 nm). Water-soluble tetrazolium salt (WST)-1 assay showed that both GPs were nontoxic to HCE cells. The fluorescence intensity of HCE cells cultured with cationic GPs conjugated with a fluorescent dye was higher than that of the anionic GP-treated HCE cells. *In vivo* examination showed no serious irritation to the rabbit eyes. Furthermore, corneal thickness and ocular pressure in the eyes of the treated rabbits were similar to those in the eyes of normal rabbits. Microscopic examination of corneal cryosections showed widely distributed fluorescent nanocarriers, from the anterior to the posterior part of the cornea of the GP(+) group, and higher fluorescence intensity in the GP(+) group was also observed. In conclusion, GPs as cationic colloidal carriers were efficiently adsorbed on the negatively charged cornea without irritating the eyes of the rabbits and can be retained in the cornea for a longer time. Thus, GPs(+) have a great potential as vehicles for ocular drug delivery.

1. Introduction

The eye poses unique challenges for drug delivery. The main objective of ocular therapeutics is to provide and maintain adequate concentration of the drug at the target site. Most ocular diseases are treated with topical application of solutions administered as eye drops. The major disadvantages of this dosage form include (i) poor ocular drug bioavailability because of the anatomical and physiological constraints of the eye that limit drug retention, (ii) pulse-drug entry with high variation in dose, (iii) nasolacrimal duct drainage, which causes systemic exposure, and (iv) poor entrance to the posterior segments of the eye because of the lens-iris diaphragm [1, 2]. The above disadvantages result in clearance of 90% of the eye drops within 2 min, and only 5% of the administered dose permeates to the eye [3].

Most efforts in ocular delivery have been focused on increasing the corneal retention of drugs with the final goal

of improving the efficacy of treatments for different ocular diseases. These attempts include the use of colloidal drug delivery systems such as liposomes [4], nanoparticles [5–8], and nanospheres [9]. The results of different studies showed the potential of nanoparticles for either gene or drug delivery for ophthalmic application. Nanoparticles are able to encapsulate and protect the gene/drug against degradation, improve tolerance, and increase corneal uptake and intraocular half-lives [10]. Gelatin nanoparticles (GPs) were selected for topical delivery because of their unique properties such as biocompatibility and biodegradability [11]. Moreover, the source of gelatin, collagen, which is the major constituent of the corneal stroma, has been used for ophthalmic applications [12].

Although several studies have examined the use of GPs for gene/drug delivery [13–16], few studies have examined the use of GPs for ocular delivery. Vandervoort examined GPs encapsulated pilocarpine or hydrocortisone for topical ophthalmic delivery [17]. Vandervoot characterized the different forms of GPs and reported the rates of drug release from these GPs, but they did not perform *in vitro* or *in vivo* tests. *In vivo* administration of GPs loaded with plasmid DNA showed significantly higher expression of MUC5AC in the conjunctiva than that in untreated controls, and naked plasmid DNA encapsulated in GPs was beneficial for ophthalmic gene delivery [18]. These results show that GPs may be effectively used as vehicles for topical administration to the eyes.

The cornea and conjunctiva possess negative surface charges, and it is expected that cationic colloidal nanoparticles may penetrate through the negatively charged ocular tissues more efficiently than anionic carriers [19]. To determine the importance of these characteristics in the interaction of nanoparticles with the cornea, we prepared GPs with a positive and negative charge for ocular delivery. The GPs with different charge were selected for ocular drug delivery. We examined the particle size, polydispersity index (PDI), shape, and surface charge and cytotoxicity of the GPs. Fluorescently labeled GPs were used in *in vitro* and *in vivo* experiments to observe the distribution of the particles in the eyes of rabbits. In addition, the central corneal thicknesses and intraocular pressure (IOP) of rabbits were also examined to confirm the influence of nanoparticles in rabbit eyes.

2. Materials and Methods

2.1. Reagent and Chemicals. Gelatin type A (derived from porcine skin, bloom 175), gelatin type B (derived from bovine skin, bloom 225), 25% glutaraldehyde (GA) solution, and acetone were purchased from Sigma-Aldrich (MO, USA). Dulbecco's modified Eagle's medium (DMEM)/F12 (1 : 1), fetal bovine serum (FBS), insulin, trypsin-EDTA, penicillin/streptomycin, and phosphate-buffered saline (PBS) were obtained from Gibco/BRL (MD, USA); epithelial growth factor (EGF) was acquired from Pepro Tech (Rocky Hill, NJ, USA). Tetramethyl rhodamine succinyl (TAMRA-NHS) ester and rabbit anti-zona occludens (ZO-1) polyclonal antibody were obtained from Invitrogen (CA, USA). The Quick Cell Proliferation Assay Kit II was got from BioVision (CA, USA). A Live/Dead Kit was purchased from Molecular Probes (OR, USA). Single-well cell inserts (PET) were obtained from Millipore (MO, USA). All other chemicals were of reagent grade and obtained from Sigma-Aldrich.

2.2. Preparation of GPs. The GPs were prepared by a two-step desolvation method as described previously with some modifications [20, 21]. Type A and type B gelatin solution (5 wt%) initially underwent desolvation by addition of excess quantity of acetone. Then, the gelatin deposited was redissolved in water. The pH of the type A gelatin solution was adjusted to 2.5 and that of type B was adjusted to 11. Acetone was added in a dropwise manner to form nanoparticles. At the end of the process, 250 μL of 8% GA solution was used as a crosslinking agent for preparing nanoparticles, and the solution was stirred for 12 h at 1000 rpm. The remaining organic solvent was evaporated using a rotary evaporator (EYELA, Tokyo, Japan), and the resultant nanoparticles were stored at 4°C for further examination.

2.3. Characterization and Measurement of Different Parameters of the GPs. The size and zeta potential of the GPs were analyzed using photon correlation spectroscopy (PCS) and laser Doppler anemometry, respectively, using a Zetasizer, 3000 HS (Malvern Instruments, UK). Each batch was analyzed in triplicate. The morphology of the nanoparticles was obtained by scanning the dried particles deposited on a flat surface with a cantilever probe model AC240 (Olympus, USA) using tapping mode in an atomic force microscope (AFM; Asylum Research, MFP-3DTM, USA).

2.4. Human Corneal Epithelial Cells Culture. The SV40-immortalized human corneal epithelial (HCE) cell line was kindly gifted by Dr. Ko-Hua Chen (Taipei Veterans General Hospital, Taiwan). The HCE cells were cultured in DMEM/F-12 supplemented with 5% FBS, 100 U/mL penicillin, 0.1 mg/mL streptomycin, 10 ng/mL EGF, 0.5% DMSO, and 5 μg/mL insulin. The cells were cultured at 37°C in a 5% CO_2-95% air atmosphere. Media were changed every other day, and the cells were observed daily under a phase contrast microscope.

2.4.1. Evaluation of Cytotoxicity of GPs. The cytotoxicity of the GPs was examined in the HCE cells using the Quick Cell Proliferation Assay Kit II (BioVision). The cells were seeded onto 96-well plates (5×10^3 cells/well) about 16 h before the experiment. The HCE cells were incubated with different concentrations of the GPs (500 to 0.1 μg/mL) for 2 h. Then, the culture medium was discarded, and 0.2 mL water-soluble tetrazolium-8 (WST-8) working solution was added to each well. WST-8 is reduced by dehydrogenases in the living cells to produce a yellow colored product (formazan). After incubation for 4 h, 100 μL of the working solution was quantitatively assessed using a SpectraMAXM5 spectrophotometer (Molecular Devices, CA, USA) at a wavelength of 450 nm. The reference wavelength was set at 650 nm. The cells were stained with a live/dead stain (Molecular Probe) to observe

cell viability. The live cells emit green fluorescence, and the dead cells emit red fluorescence. Images were acquired using an inverted fluorescence microscope (Nikon, TiS, Japan) and were analyzed using Nikon NIS Element software.

2.4.2. Evaluation of Transepithelial Electrical Resistance. About 3×10^5 HCE cells/cm^2 were seeded on PET inserts with a 0.4-μm pore size (Millipore, MA, USA), and the medium was replenished every other day. Resistance across the insert membrane was measured using the STX2 electrode set (World Precision Instruments [WPI], Florida, USA). The transepithelial electrical resistance (TEER) of cells grown on filters was measured with an epithelial voltohmmeter (EVOM, WPI). Cells were used only if their TEER was more than 100 V/cm^2. The suspension of GPs (100 μg/mL) was added into the media of the insert well. The electrode set was inserted in both the chambers for the indicated times. The TEER was calculated from the measured resistance and normalized using the area of the monolayer (ohms per square centimeter). The background TEER of blank insert filters was subtracted from the TEER of the cell monolayers. Chitosan nanoparticles (CNP) were used as the positive control because of their capacity to disrupt the tight intercellular junctions [22]. The size of the CNP was about 180 nm and their zeta potential showed a positive charge (20 mV).

2.4.3. Western Blotting. The HCE cells were lysed to extract the cellular protein, and their absorbance was measured at OD 260/280 nm before use. Equal amounts of protein (approximately 10 μg) were separated using 6% sodium dodecyl sulfate polyacrylamide gel electrophoresis. Then, the proteins were transferred onto nitrocellulose membrane, and the membranes were blocked in 5% nonfat powdered milk in Tris-buffered saline (TBS) and 0.05% Tween. The membranes were incubated with the primary antibody (ZO-1 at 1 : 3000 overnight at 4°C) followed by incubation with the appropriate secondary antibodies (horseradish peroxidase [HRP]-conjugated anti-rabbit antibody at 1 : 10,000 for 1 h at room temperature). α-Tubulin was used as the internal control. Bands were visualized using an enhanced chemiluminescence reagent and exposed to a Fujifilm LAS1000 Intelligent Dark Box and captured digitally.

2.4.4. Cellular Uptake Study. GPs with positive or negative charge were labeled with red fluorescence via being conjugated with TAMRA-NHS ester (Invitrogen) according to the method described by the manufacturer. The concentration of the fluorescent dye (TAMRA) in the GPs (100 μg/mL) was 0.35 μg/mL. In addition, we examined the culture medium with the dye concentration equal to that in the aqueous formulation. The fluorescent GPs (100 μg/mL) were cultured with HCE cells for 2 h; subsequently, the medium was removed, and the cells were washed twice using PBS. Subsequently, 0.2 mL of cell lysis solution (50 mM Tris-HCl, pH 7.6, 300 mM NaCl, and 0.5% Triton X-100) was added to the cell pellets, and they were maintained for 2.5 h on ice with frequent vortexing. Then, the cells lysate was collected into Eppendorf tubes and centrifuged at 12,500 rpm for 20 min. The cell suspension (100 μL) was added to a 96-well plate and the OD was measured at an excitation wavelength of 546 nm and an emission wavelength of 576 nm by the microplate spectrophotometer (SpectraMAXM5) under fluorescence mode.

2.5. Preliminary Animal Study. Male New Zealand rabbits weighing 2.5–3.5 kg and with no signs of ocular inflammatory or gross abnormalities were used. The *in vivo* experimental protocol was approved by the Institutional Animal Care and Use Committee of the Taipei Medical University (IACUC Approval No. LAC-100-0165). The animals were housed in standard cages in a light-controlled room and were given food and water *ad libitum*. We used 9 rabbits for measurement at each time point, and during the experiments, the rabbits were allowed to move their heads freely, and their eye movements were not restricted.

2.5.1. In Vivo Tolerance. Positively charged GPs conjugated with the fluorescent dye (GP [+] TAMRA) were used in this study. We administered 50 μL of sterilized GP(+) TAMRA in the lower conjunctival sac of the right eye of rabbits. The rabbits simultaneously received 50 μL of TAMRA in PBS in their left eye. The same volume of PBS was administered to another group of rabbits as control. This irritation test was performed using a clinical evaluation scale of 0 (absence) to 3 (highest) of discomfort, discharge, cornea/conjunctival chemosis, or redness as described in Table 2 using a modification of the scoring system established in the 2002 Organization for Economic Cooperation and Development guidelines for ocular irritation testing [4, 23]. The test was performed on 5 eyes of each group; the test was performed in 3 eyes in the PBS-treated (control) group. Each animal was observed and tested at 0.5, 2, 4, and 16 h after instillation.

2.5.2. Clinical Observations. At each study point, we measured the intraocular pressure (IOP) using a Schiotz tonometer (AMANN Ophthalmic Instruments, Liptingen, Germany) calibrated according to the manufacturer's instructions. For determination of IOP, 5 readings were taken on each eye alternating between the left and right eyes, and the mean was calculated [24]. Central corneal thickness (CCT) was determined using an ultrasonic pachymeter (DGH Technology, Exton, PA, USA) with a hand-held solid probe [25]. During the measurements, the probe tip of the pachymeter was held perpendicular to the central cornea. Averages of 10 readings were recorded. An ophthalmic table slit lamp (Topcon Medical Systems Inc., NJ, USA) was used to observe and record the anterior segment. The rabbits were killed 16 h after administration of the eye drops. The eyeballs were harvested and fixed in 3.7% formaldehyde.

2.5.3. Fluorescence Quantification. The rabbits were killed at 0.5, 2, 4, and 16 h after the last instillation. Eyeballs were harvested and cleaned using PBS. Fluorescent GPs in the eyes were quantified using an *in vivo* imaging system (IVIS Imaging System 200 Series; Xenogen, USA). The relative intensity of fluorescence in the eyes was equivalent to the concentration of fluorescent nanoparticles. The fluorescence

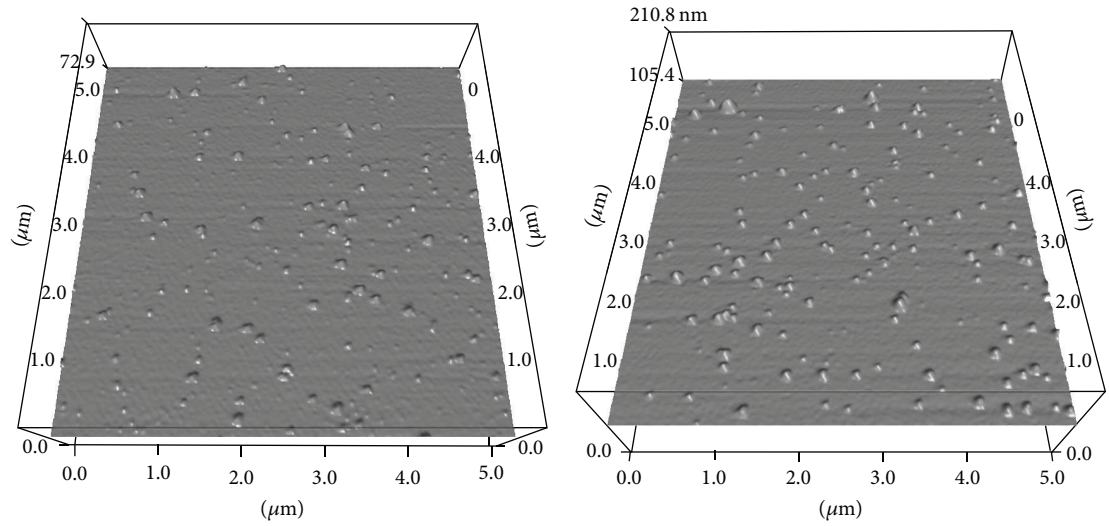


Figure 1: Morphology and size of charged GPs with (a) positive or (b) negative charge. Image acquired by atomic force microscopy.

Table 1: Size and zeta potential of gelatin nanoparticles ($n = 3$).

	Size (nm)	Zeta (mV)
GPs(+)	180.6 ± 45.7	33.4 ± 10.9
GPs(−)	230.7 ± 84.6	−44.2 ± 7.2

intensity of the PBS-treated group was used as the background value. The quantified area was restricted to the cornea, and the fluorescein signal was calculated ($n = 5$).

2.5.4. Distribution of GPs in the Cornea Observed Using Confocal Laser Scanning Microscopy. After the fluorescence intensity was quantified, the cornea was excised from the eyeball and separated into 2 sections. One section was directly mounted on a glass slide and examined under a microscope without additional processing of the tissue. A 5-μm cryosection was prepared using the other section from the apical to the lateral end of the cornea. All cornea samples were analyzed with a confocal microscope (Nikon, A1, Japan).

2.6. Statistical Analysis. Experiments were performed at least in triplicate, and the results were reported as mean ± standard deviation (SD). All data were analyzed with the Student's t-test or one-way analysis of variance (ANOVA). Statistical significance was considered at a level of $P < 0.05$.

3. Results and Discussion

In this study, we prepared charged GPs and performed *in vitro* and *in vivo* studies. The rabbit cornea model was used to determine the retention of the cationic GPs because of the similarity of this model with that of the human cornea [10, 26].

3.1. Characterization of GPs. GPs can be prepared using type A or type B gelatin to obtain positively or negatively charged nanocarriers (Table 1). The size of GPs prepared using type A gelatin was approximately 180.6 ± 45.7 nm. The size of the negatively charged GPs (prepared using type B gelatin) was 230.7 ± 84.6 nm; these nanoparticles were larger and more widely distributed than GPs prepared using type A gelatin. The zeta potential of GPs prepared using type A and type B gelatin was 33.4 ± 10.9 mV and −44.2 ± 7.2 mV (Table 1). The nanoparticles prepared using type A gelatin had a positive surface charge and were abbreviated as GP(+), and those prepared using type B gelatin were negatively charged and were abbreviated as GP(−). The nanoparticles of both types observed under the AFM showed a smooth and ball like structure (Figure 1). The particle size was about 200 nm, which was consistent with the findings of photon correlation spectroscopy (PCS). Type A and type B gelatin were prepared using by different processes by extracting gelatins from collagen [11]. The amount of free carboxyl or amino groups was different in both types of gelatin. At pH 6~7, however, type A gelatin has a positive net charge, while type B gelatin is negatively charged [17, 27]; thus, the zeta potential of these gelatins may also be different. The positively charged GPs (GP+) may have electrostatic attraction with the negatively charged corneal epithelial cells, which is more preferred in ocular drug delivery.

3.2. Cytotoxicity of GPs. An important aspect of the development of new carrier for drug/gene delivery is its safety of interaction with the target cells. The biocompatibility of the newly developed materials should be examined to determine their potential for ophthalmic use. In this study, we evaluated the cytotoxicity of GPs in the HCE cell line by measuring their metabolic activity. The percentage of viable cells in the treated group versus nontreated group (culture medium) is

Table 2: Grading system of the macroscopic signs in the *in vivo* tolerance study for the colloidal system tested [23].

Grade	Discomfort	Cornea	Conjunctiva	Discharge	Lids
0	No reaction	No alterations	No alterations	No discharge	No swelling
1	Blinking	Mild opacity	Mild hyperemia Mild edema	Mild discharge without moistened hair	Mild swelling
2	Enhanced blinking Intense tearing Vocalizations	Intense opacity	Intense hyperemia Intense edema Hemorrhage	Intense discharge with moistened hair	Obvious swelling

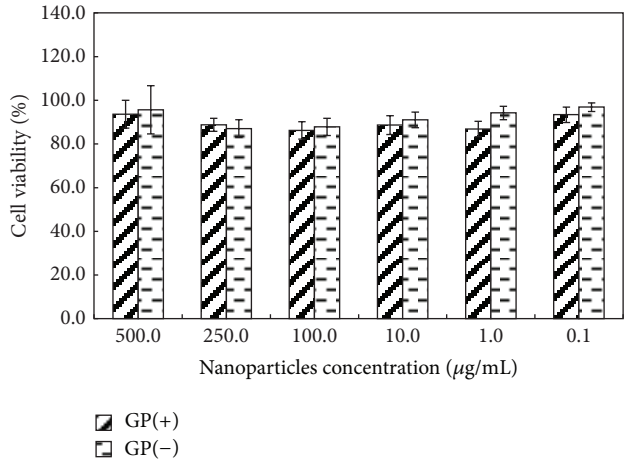


Figure 2: Results of WST-1 assays of human corneal epithelium (HCE) cells after incubation with 2 kinds of gelatin nanoparticles for 2 h. (GP+, GPs with positive surface charge; GP−, GPs with negative charge. Data were analyzed using the Student's t-test and are presented as mean ± standard deviation (SD); $n = 6$, $^{*}P < 0.05$).

shown in Figure 2. No significant difference was observed in cell viability even after treatment with 500 μg/mL of GPs for 2 h. The images of HCE cells labeled using the live/dead stain are shown in Figure 3; the live cells emit green fluoresce and the dead cells emit red fluoresce. Large percentage of live cells was observed in the control group (Figure 3(a)), and nearly all the HCE cells were viable after coculturing with GP(+) or GP(−) for 2 h (Figures 3(b) and 3(c)). HCEs were viable and only a few dead cells were observed after culturing with GPs, which indicated that GPs had adequate safety for application to the ocular surface. de la Fuente et al. cultured HCE cells with hyaluronic acid-CNP for 1 h and showed that this treatment had no effect on cell viability [7]. The viability of HCE cells treated with cationized gelatin nanovector hybrid with chondroitin sulfate or dextran sulfate for 72 h was not significantly different from that nontreated HCE cells [18]. These results indicate that GPs are not toxic to HCE cells after short-term or long-term exposure.

3.3. Alteration in the TEER across the Tight Junction in HCE Cells by GPs Treatment. The presence of tight junctions between epithelial cells prevents the flow of the fluids and the movement of molecules and ions between cells [22]. The epithelial membrane provides a significant barrier to the free diffusion of substances from the cornea to the anterior chamber. The barrier integrity of these monolayers can be measured directly by measuring the TEER of HCE cells. The TEER of HCE monolayers cultured with charged GPs (GP+/GP−) slightly increased to 110% ± 12.7% (% against initial) and then returned to normal (Figure 4(a)). No significant difference was observed in the TEER of HCE cells treated with GPs(+) or GP(−) after 96 h. However, cells treated with CNP showed a marked decrease in the TEER (70% decrease), which showed that the barrier integrity of the HCE monolayer was changed by CNP treatment. The CNP-treated cells showed loss of ZO-1, but no variation in the 2 GPs groups (Figure 4(b)). Chitosan has been widely used for ocular drug delivery [5, 28–30] because of its mucoadhesive property. Chitosan disrupts the tight intercellular junctions and results in loss of membrane-associated ZO-1, thus increasing the permeability of the epithelium [22]. The anterior part of the eye is constantly exposed to the external environment and thus is vulnerable to a wide range of microorganisms; further, its moist mucosal surface makes the cornea particularly susceptible to attack. The barrier to avoid microorganism invasion depends on the integrity of the tight junctions in the cornea. The tight junctions between the neighboring epithelial cells prevent the free diffusion of hydrophilic molecules across the epithelium by the paracellular route [31]. However, CNP increases the drug concentration in the cornea via intracellular (uptake by the cells) and intercellular (opening the tight intercellular junction) routes [31]. Previous study showed that the tight junction reclosed may be impeded by the unremoved chitosan residue on the surface of the Caco-2 cells [22]. Therefore, a similar phenomenon may be observed in the corneal epithelial cells causing continuously

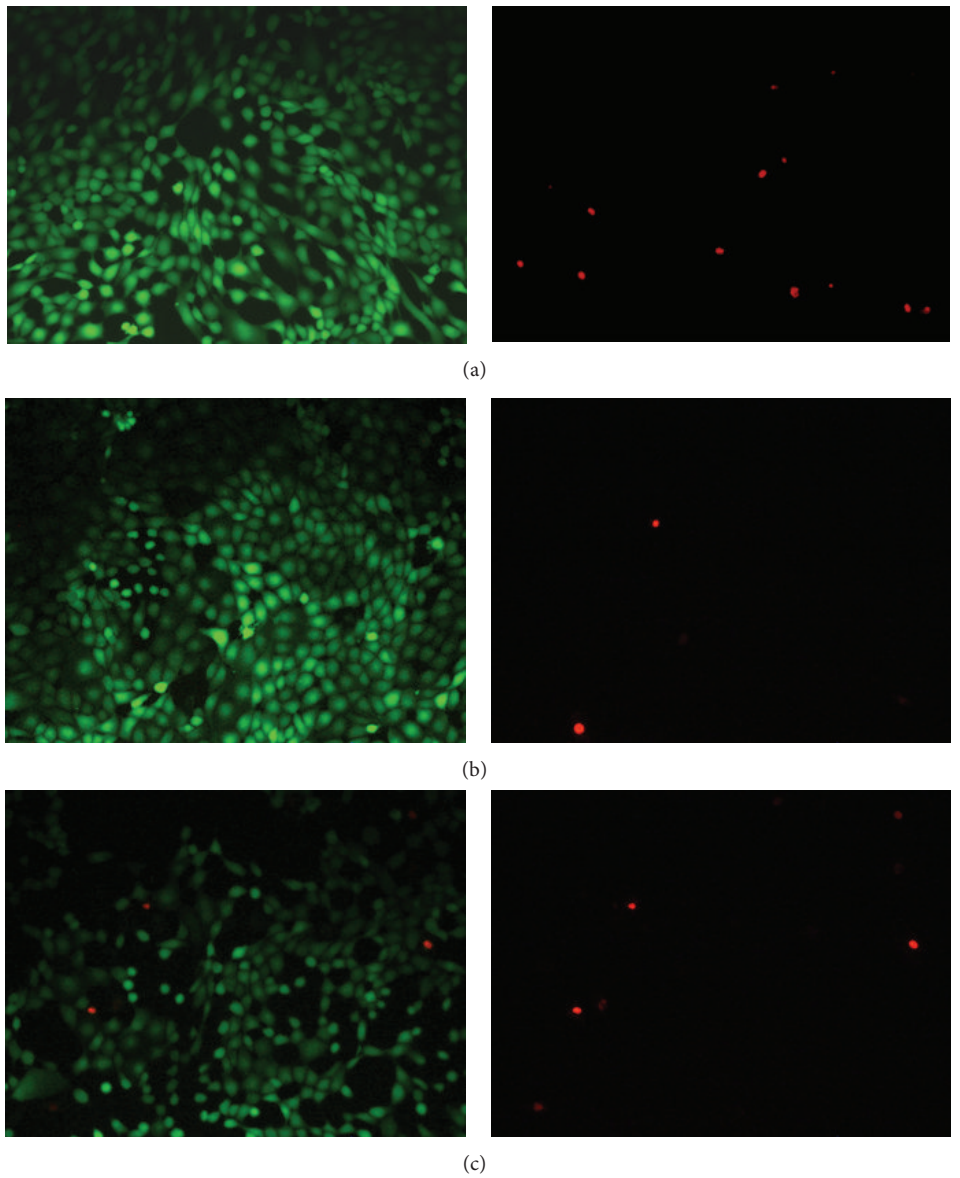


Figure 3: Fluorescent photomicrographs of human corneal epithelial (HCE) cells cultured with (a) culture medium, (b) GP(+), and (c) GP(−) at a concentration of 100 μg/mL for 2 h. The polyanionic dye calcein-AM is well retained within the live cells, which produced intense uniform green fluorescence in the live cells. (Magnification: 40x); GP: gelatin nanoparticles.

open of the tight junction. Therefore, a risk for using CNP for ocular drug delivery is increasing the microorganism invasion to the cornea via disruption of cornea tight junction. But, there is no risk for tight junction disruption by GPs.

3.4. Intracellular Content. We examined the intracellular accumulation of the charged GPs in the HCE cells. We examined internalization of fluorescence-labeled GPs by measuring the fluorescence in the cell lysates. Cationic or ionic GPs conjugated with TAMRA were added into the culture medium. The intracellular fluorescence of the cell lysates in the GP(+) group at 10, 30, and 60 min was higher than that of the GP(−) (Figure 5). After 60 min, the OD value of the GP(+) group was much higher than that of the GP(−) group ($P < 0.05$). This finding is consistent with previous study, which showed that cationic nanoparticles could increase the stability of the nanoparticle system and improve the interaction between the particles and the eye surface and thus increase the transfection efficiency [18, 32].

3.5. Tolerance and Clinical Evaluation. Gelatin is commonly used in the preparation of capsules, and GPs are widely investigated for drug/gene delivery. However, few studies have examined the safety and tolerance of GPs for ocular drug delivery. We performed an irritation test on rabbits after single instillation of 50 μL of GPs formulation. The eye treated using PBS was used as a control. Each animal was observed at 0.5, 2, 4, and 16 h after instillation. An index of overall irritation (Table 2) was calculated by summing up the total clinical evaluation scores over the observation time

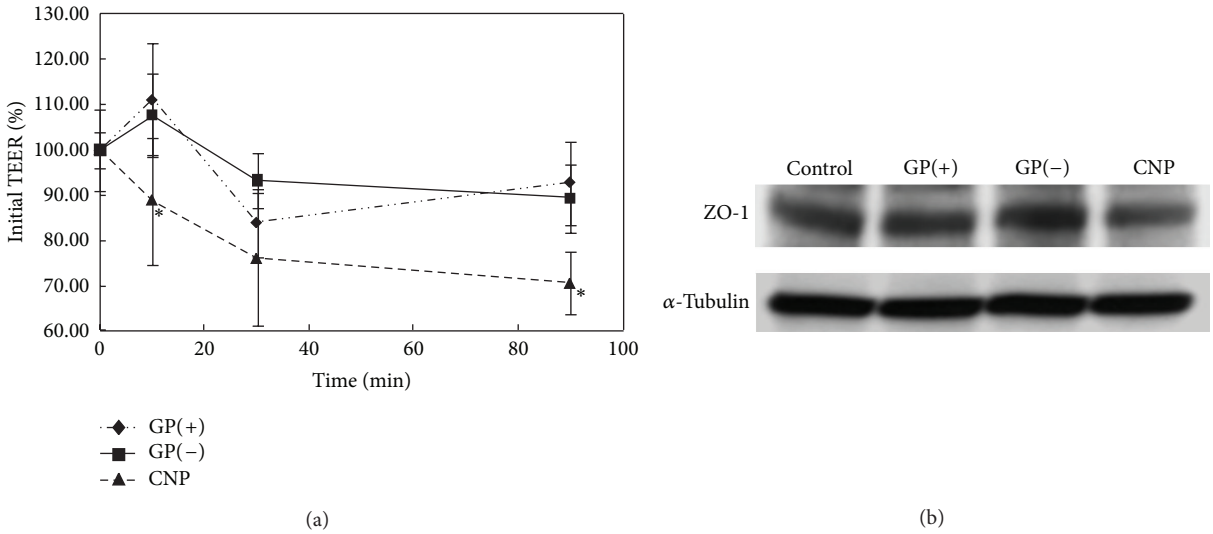


Figure 4: The transepithelial electrical resistance (TEER) assay showed recovery of human corneal epithelial (HCE) cell layer barrier after coculturing with gelatin nanoparticles (GPs), but not in chitosan nanoparticles (CNP). $n = 5$ standard error of mean (SEM), $^{*}P < 0.05$. (b) Western blot analysis of zonula occluden-1 (ZO-1) expression in HCE cells after treatment with different nanoparticles.

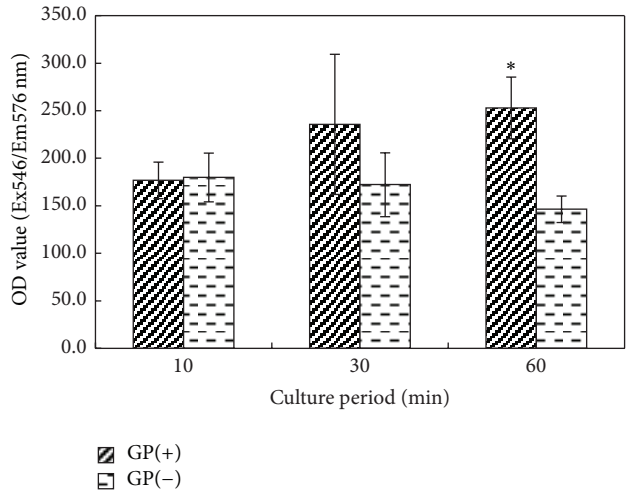


Figure 5: Nanoparticles uptaken by the human corneal epithelial (HCE) cells were evaluated by measuring the fluorescence intensity of the cell lysate. $n = 6$ standard error of mean (SEM), $^{*}P < 0.05$.

Table 3: Grading system of macroscopic signs in the *in vivo* tolerance study of the gelatin nanoparticles.

	Control*		Free TAMRA				GP(+) TAMRA			
Time	0.5 h	4.0 h	0.5 h	2.0 h	4.0 h	16.0 h	0.5 h	2.0 h	4.0 h	16.0 h
Grade										
Discomfort	0	0	0	0	0	0	0	0	0	0
Cornea	0	0	0	0	0	0	0	0	0	0
Conjunctive	0	0	0	0	1	0	0	0	1	0
Discharge	0	0	0	0	1	0	1	0	0	0

*$n = 3$.
GP: gelatin nanoparticles; TAMRA: tetramethyl rhodamine.

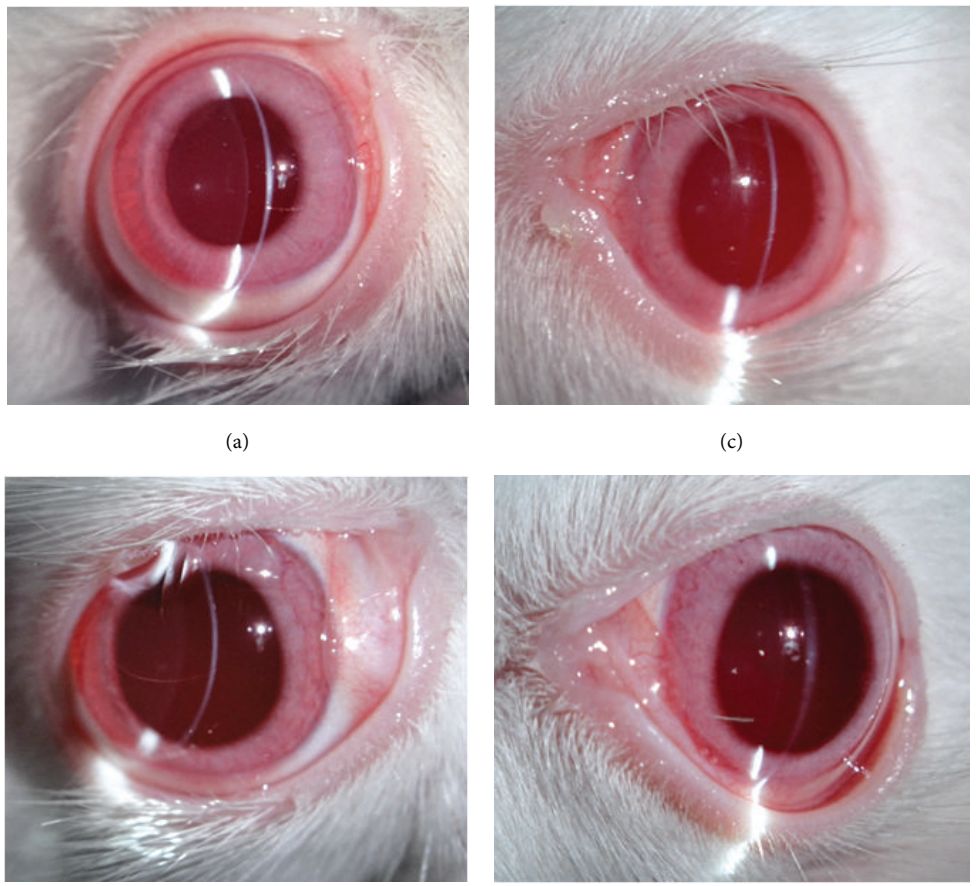


Figure 6: The appearance of rabbit eyes topically treated with 100 μL of free tetramethyl rhodamine succinyl (TAMRA) solution: (a) 0.5 h and (b) 16 h after treatment; treated with GP(+)TAMRA solution (100 μL): (c) 0.5 h and (d) 16 h after the application.

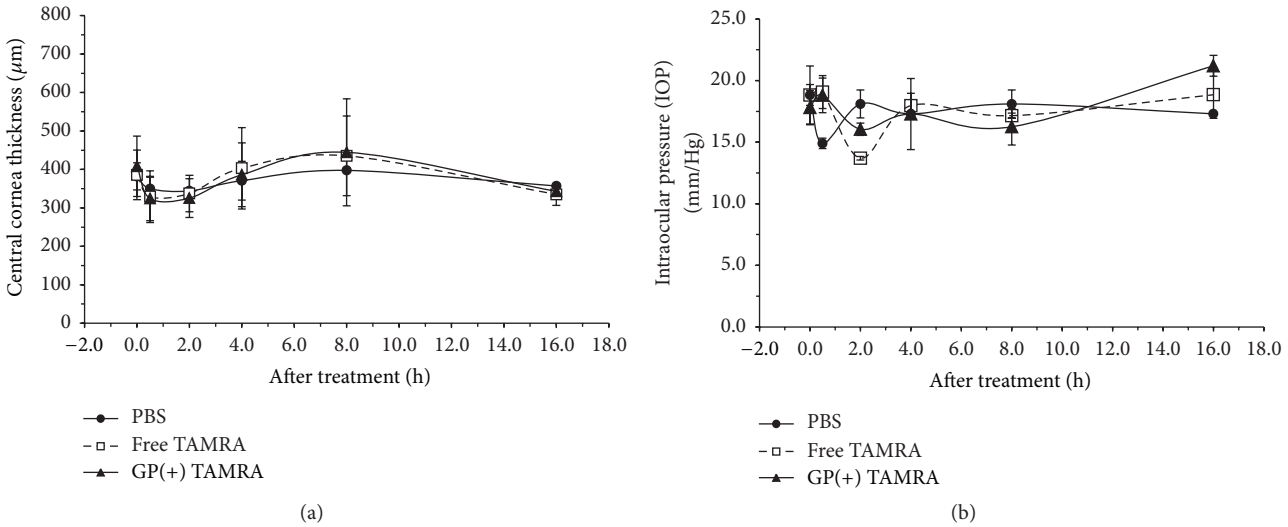


Figure 7: (a) Measurements of central corneal thickness (CCT) and (b) intraocular pressure (IOP) after treatment with eye drops containing TAMRA solution or GP(+)TAMRA. An asterisk indicates statistically significant differences ($^*P < 0.05$; $n = 5$) compared to control (PBS-treated rabbits, $n = 3$).

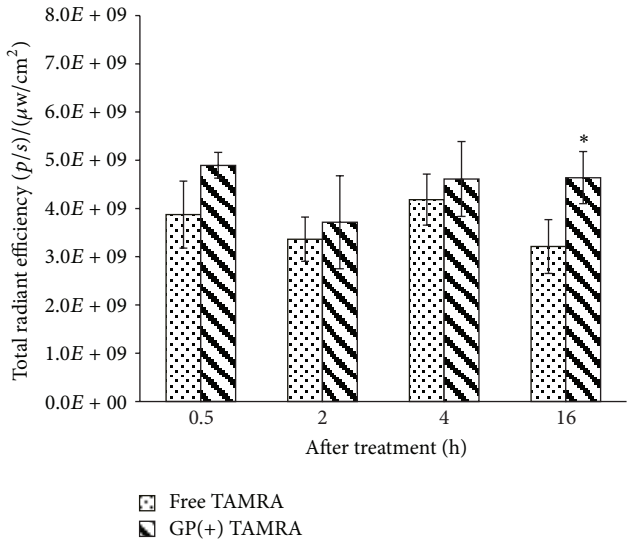
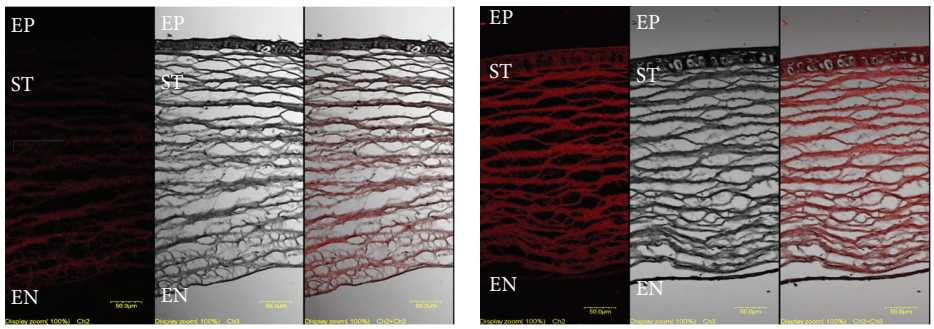


FIGURE 8: *Ex vivo* fluorescence imaging of the eyes of rabbits treated with fluorescent dye for different time periods: (a) TAMRA solution and (b) GP(+)TAMRA. $n = 5$ standard error of mean (SEM), $^{*}P < 0.05$.



(a) (b)

FIGURE 9: ((a), (b)) are images from cryosections of the cornea treated with the free dye and GP(+) fluorescence dye. Free dye: TAMRA/PBS solution; GP(+) dye: GP(+) with TAMRA conjugation (red). Scale bar: 50 mm. EP: corneal epithelium; ST: corneal stroma; EN: corneal endothelium. After treatment for 0.5 h.

points; the results are shown in Table 3. Very slight redness of the conjunctiva was observed in the eyes treated with free TAMRA solution and in GP(+) TAMRA-treated eyes at 4 h, but no chemosis was observed after treatment in other groups and at other time points. No differences were observed in the ocular tissue of rabbits treated with free and GP(+) TAMRA after 0.5 and 16 h (Figure 6). Treatment with GP(+) was safe and caused no irritation to the eyes of the rabbits. Previous studies have shown that the rabbit eye is more sensitive than the human eye and has a longer time for epithelial repair [10, 33]. Therefore, it is reasonable to expect that charged GPs are well tolerated by the human eye.

One of the risk factors involved in eye disease is increased IOP, which leads to apoptosis and loss of retinal ganglion cells [34]. Therefore, we examined the changes in the IOP and corneal thickness after eye drop treatment to confirm the safety of GPs for ocular drug delivery. The effects of instillation of free TAMRA and GP(+) TAMRA eye drops on corneal thickness and IOP in rabbits are shown in Figure 7. Compared to the control groups (328 ± 21 μm), the groups treated with free TAMRA and GP(+) TAMRA eye drops showed a decrease in the corneal thickness after 0.5 h (297 ± 4 μm; 292 ± 6 μm) and 2 h (304 ± 5 μm; 290 ± 5 μm) (Figure 7(a)). After 4 h of treatment, the corneal thickness in the treated group was almost the same as that in the control group. The mean baseline of IOP values ranged from 16 to 20 mmHg (Figure 7(b)). The IOP decreased immediately after treatment with PBS. The IOP returned to the baseline level within 2 h. In addition, the IOP decreased at 2 h even after treatment with free TAMRA and GP(+) TAMRA. However, the IOP in these 2 groups returned to the normal range after 4 h (Figure 7(b)). The corneal thickness and IOP did not change significantly after treatment with GP(+) TAMRA suspension.

3.6. Fluorescence Examination to Determine the Distribution of GPs in the Eyes. The amount of fluorescent nanoparticles in the cornea at different time points acquired using the IVIS spectrum imaging system is shown in Figure 8. The number of fluorescent spots obtained after treatment with

GP(+) TAMRA was greater than that obtained treatment with free TAMRA/PBS at 0.5, 2, 4, and 16 h. The accumulation of the fluorescent dye differed significantly between the free TAMRA and GP(+) TAMRA treated group at 16 h after treatment. After IVIS examination, the cornea was removed and a cryosection was prepared for examination under a confocal microscope. The distribution profile of the fluorescent dye in the cornea of rabbits after administration of the eye drops is shown in Figure 9. The cross-section of the cornea from the epithelium, stroma to endothelium layer, was observed under the same magnification. The cornea treated using the free TAMRA solution showed a weak fluorescent signal located in the posterior region (Figure 9(a)). The cornea treated with GP(+) TAMRA showed a strong fluorescent signal in the entire cornea and (Figure 9(b)). Moreover, the fluorescence quantification of the cornea treated with GP(+) TAMRA increased by 4-fold compared to that treated with TAMRA/PBS solution, which indicated that the dye encapsulated in the GPs could be retained in the cornea for a longer time and was distributed uniformly across the entire cornea. Solid lipid nanoparticles (SLNs) and CNP are retained for a longer time on the corneal surface probably because of their small size, and further characterization of nanoparticles would help in determining the transcorneal absorption [5, 10]. Hyaluronic acid coated poly-3-caprolactone nanospheres achieved high levels of cyclosporine A (CyA) in the cornea, which was 10-fold higher than that was achieved with CyA solution in castor oil [35]. In our study, the levels of GPs in the corneas treated with GP(+) TAMRA were higher than those in the cornea treated with free TAMRA at each time point, which indicated a longer retention time (16 h) of GP(+) TAMRA compared to that of free TAMRA. Our *in vivo* results might be explained on the basis of the prolonged retention in the precorneal area and cornea because of the small size of GP(+) (180 nm) and also in the uptake/internalization of GP(+) into the corneal epithelium.

4. Conclusion

The aim of this study was to confirm whether cationic GPs could be used for topical application, and this was examined in rabbit eyes. Positively charged GPs were prepared with a size of about 180 nm. GPs are nontoxic to HCE cells and had no influence on the tight intercellular junctions. The corneal thickness slightly decreased 0.5 h after treatment and then returned to normal. The IOP showed variation in the normal range after treatment with GPs. GPs showed retention of the fluorescent dye in the cornea for the prolonged period, which is beneficial to maintain the dose in the therapeutic range. Therefore, dye/drug/gene encapsulated in cationic GPs nanoparticles is promising new medicines for ocular disease.

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