Supporting Information

Gelatin-Functionalized Mesoporous Silica Nanoparticles with Sustained Release Properties for Intracameral Pharmacotherapy of Glaucoma

Yu-Te Liao,[a] Chih-Hung Lee,[b] Si-Tan Chen,[b] Jui-Yang Lai,*[b,c,d] and Kevin C.-W. Wu*[a,e,f]

a: Department of Chemical Engineering, National Taiwan University, No. 1, Sec. 4, Roosevelt Road, Taipei 10617, Taiwan.
b: Institute of Biochemical and Biomedical Engineering, Chang Gung University, No. 259, Wen-Hwa 1st Road, Kwei-Shan, Taoyuan 33302, Taiwan.
c: Department of Materials Engineering, Ming Chi University of Technology, No. 84, Gungjuan Road, Taishan, New Taipei City 24301, Taiwan.
d: Department of Ophthalmology, Chang Gung Memorial Hospital, No. 5, Fushing Street, Kwei-Shan, Taoyuan 33305, Taiwan.
e: Division of Medical Engineering Research, National Health Research Institutes, 35 Keyan Road, Zhunan, Miaoli County 350, Taiwan.
f: Department of Chemistry, Chung Yuan Christian University, Chung Li 32023, Taiwan.

*E-mail: kevinwu@ntu.edu.tw, jylai@mail.cgu.edu.tw

Materials and Methods

1. Chemicals

Hexadecyl trimethyl ammonium bromide (CTBr), tetraethyl orthosilicate (TEOS), gelatin (type A, 300 bloom), pilocarpine nitrate, *N*-hydroxysuccinimide (NHS), *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC), matrix metalloproteinase-2 (MMP-2) and 4-morpholineethanesulfonic acid (MES) were purchased from Sigma-Aldrich without further purification. Phosphate buffered saline (PBS), fetal bovine serum (FBS),

Dulbecco's Modified Eagle Medium (DMEM), L-glutamine, sodium bicarbonate, antibiotic/antimycotic (A/A) solution and (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) (MTT) were purchased from Biological Industries. Ammonium hydroxide solution (NH_4OH, 28%), hydrochloride acid (HCl, 35%), monobasic potassium phosphate dimethyl sulfoxide (DMSO) and methanol were purchased from J.T. Baker. Deionized water used was purified with a Milli-Q system (Millipore, Bedford, MA, USA). Balanced salt solution (BSS, pH 7.4) was obtained from Alcon Laboratories (FortWorth, TX, USA).

2. Synthesis of mesoporous silica nanoparticles (MSNs) and gelatin covered MSNs (GM)

The MSNs were synthesized via the process of sol-gel and self-assembling. 1 g of CTABr and 3.5 mL of NH_4OH (28%) were dissolved in 450 mL of D.I. water at 45 °C. 33.8 mL of TEOS contained ethanol (0.2 M) was added into above solution. The mixed solution was stirred for 5 h at 300 r.p.m.. Then, 13.8 mL of TEOS contained ethanol (1 M) was added into solution and stirred for another 1 h at 450 r.p.m.. After that, the stirring rate was kept at 300 r.p.m. for 1 day. The as-synthesized MSNs were collected by filtration. 1 L of D.I. water and 1 L of methanol were used to wash the cake sequentially. The powder of as-synthesized MSNs were refluxed in 300 mL of methanol containing 6 mL of HCl (37 wt%) to remove the surfactant stocking inside the tunnel of MSNs for 12 h. Finally, MSNs were collected by filtration and washed with 2 L of methanol, later freeze-dried and stored for use.

To synthesize gelatin covered MSNs (GMs), different ratio of gelatin to MSN were mixed together in MES buffer (10 mM, pH 5) at 37 °C for 2 h. EDC and NHS were added into above

solution and stirred for 10 h at 37 °C. The weight ratio (mg) between gelatin, EDC and NHS was 10:13.5:2. The GMs were collected by centrifugation and freeze-dried for use.

3. Synthesis of drug loaded MSNs (p/MSN) and drug loaded, gelatin covered MSNs (p/GM)

10 mg of MSNs and 27.1 mg of pilocarpine were stirred in 10 mL of MES buffer solution (pH 5, 10 mM) for 2 days. The p/MSNs were collected by centrifugation and freeze-dried for use. The procedure to synthesize p/GM is followed by the procedure to synthesize p/MSN. The as-synthesized p/MSN was mixed with gelatin and stirred for 2 h at 37 °C in MES buffer solution (pH 5, 10 mM). Different amount of EDC and NHS were then added into solution and stirred for another 10 h at 37 °C. The weight ratio (mg) between gelatin, EDC and NHS was 10:13.5:2. The p/GM were collected by centrifugation and freeze-dried for use.

The amount of pilocarpine remaining in solution was quantified by using high performance liquid chromatography (HPLC, Waters 600 equipped with Waters 2489 UV/Visible detector) with C-18 column (DiKMA). The mobile phase was 5% monobasic potassium phosphate contained water (pH was adjusted to 2.5 by 85% of phosphoric acid) mixed with methanol in the ratio of 85 to 15 (volume). The flow rate was 0.7 mL/min and eluent peak was detected by measuring absorbance at 216 nm. The calculation of encapsulation efficiency was based on the equation as shown below.

$$\text{Loading content}\left(\frac{\mu g}{mg}\right) = \frac{\text{drug input (mg) - drug in supernatant (mg)}}{\text{MSNs in suspension (mg)}} \times 1000$$

4. Characterization

The morphology of MSNs and the core-shell structure of GM was observed by transmission electron microscopy (TEM, JEOL JEM-1200EX II). The zeta potential of each sample (i.e., MSN, GM and gelatin) was measured by using Zetasizer (Malvern, Nano-ZS). The functional groups on different samples (i.e., MSN and GM) were measured with Fourier transform infrared (FTIR) spectra (Perkin Eimer, Spectrum 100). Samples used for FTIR measurements were prepared by mixing the vacuum-dried samples with KBr (KBr : sample = 100 : 1). The mixture was then ground extensively and pressed into a translucent disc. The surface area and pore size distribution of MSN and GM were measured with nitrogen adsorption/desorption (micromeritics, ASAP 2010). The calculation was based on Brunauer-Emmett-Teller (BET) theory and Barrett-Joyner-Halenda (BJH) theory. The amount of gelatin on GM was analyzed with thermal gravimetric analysis (TGA, Perkin Elmer, pyris 1).

5. *In vitro* release of drug from p/MSN and p/GM

4 mg of p/MSN and p/GM was added into microcentrifuge tube with 2 mL of buffer saline solution (BSS) containing 50 ng/mL of MMP-2 separately. The microcentrifuge tubes were shook with 300 r.p.m. at 37 °C in thermal shaker incubator. At predetermined time intervals, 0.5 mL of mixture was extracted and replaced with fresh BSS containing MMP-2 (50 ng/mL). The extracted solution was centrifuged to separate the particles and solution. The supernatant was analyzed by using HPLC with conditions mentioned in section of characterization.

6. Cell culture and MTT assay

Bovine corneal endothelial cells (BCE C/D-1b; ATCC No: CRL-2048) was purchased from the American Type Cell Collection (Manassas, VA, USA). BCE was routinely cultured in Dulbecco's Modified Eagle Medium (DMEM) containing additives (i.e. 10% of FBS, 4 mM of L-glutamine, 1.5 mg/mL of sodium bicarbonate, 4.5 mg/mL of glucose and 1% of A/A solution) and incubated at 37 °C in a humidified atmosphere with 5% CO_2. Cell viability was measured by MTT assay. Cells were cultured in 96-well microplate (10^5 cells/well) with 200 μL of DMEM containing 20% FBS for 1 day. Samples (i.e. MSN and GM) were suspended into serum-free medium to achieve different concentration. The suspension were then added into each well and incubated at 37 °C for 4 h. The medium was then removed, and each well was washed three times with PBS. After that, 200 μL of DMEM containing MTT (0.5 mg/mL) were added into each well. The plates were left stationary for 4 h. The medium was then replaced with 150 μL of DMSO to dissolve the blue crystals. The absorbance was recorded by a microplate reader at wavelength of 570 nm.

7. Animal studies

In this study, all animal procedures were approved by the Institutional Review Board of Chang Gung University (IACUC approval number: CGU13-024) and were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Twenty-four adult New Zealand white rabbits from National Laboratory Animal Breeding and Research Center (Taipei, Taiwan, ROC) weighing 3.0-3.5 kg and 16-20 weeks of age were used as animal model. All the rabbits were separated into four groups including one control and three experimental groups. Surgical operation was performed in the single eye of animals. The rabbits belonging to control group would not receive any drug. On the other hand, the rabbits belonging

to other three experimental groups would receive p/MSN, p/GM-0.05 and p/GM-0.5 by intracameral administration.

To trigger experimental glaucoma in rabbit eye, we adopted a procedure similar to Percicot et al.[1] mentioned before. Briefly, 2.5 mg/kg body weight of tiletamine hydrochloride/zolazepam hydrochloride mixture (Zoletil; Virbac, Carros, France) and 1 mg/kg body weight of xylzine hydrochloride (Rompun, Bayer, Leverkusen, Germany) were used to anesthetize the rabbits intramuscularly first. Then, 0.1 mg/mL of α-chymotrypsin was injected into the posterior chamber of the eye. The 30-gauge needle used for α-chymotrypsin injection was swept across to homogeneously distribute the enzyme throughout the posterior chamber. The needle remained in position for an additional 2 min before it was withdrawn to avoid and contact of the enzyme with the corneal endothelium. To prevent eye inflammation and pain, each operated eye received two drops of tobramycin-dexamethasone ophthalmic solution (Tobradex; Alcon-Couvreur, Puurs, Belgium) and one drop of diclofenac sodium ophthalmic solution (Voltaren; Ciba Vision Ophthalmics, Duluth, GA, USA) three times a day during the first week of follow-up experiment. In this research, the animals were considered to be glaucomatous when IOP was higher than 30 mmHg after 4 weeks of α-chymotrypsin injection.

Anterior chamber was entered with a 30-gauge needle near the limbus and injected respectively with 30 μL of p/MSN contained, p/GM-0.05 contained and p/GM-0.5 contained solution respectively. Each solution was prepared by suspending the drug contained nanoparticles into BSS with the concentration of 1 mg per 30 μL.

The therapeutic efficacy was determined based on ophthalmic evaluations performed before and immediately after drug administration. Subsequently, the bilateral eyes of 24 rabbits

were examined at predetermined time intervals for 3 weeks. The slit-lamp biomicroscopy (Topcon Optical, Tokyo, Japan) was utilized to observe the morphology of the anterior segment of the eye including corneal and lens clarity, the degree of anterior chamber activity and iris. The density of corneal endothelial cell in rabbit's eyes was measured by specular microscopy (Topcon Optical). IOP was measured by a Schiotz tonometer (AMANN Ophthalmic Instruments, Liptingen, Germany). The calibration was based on the manufacturer's instructions. IOP on each eyes was measured for five times and the mean value of IOP was calculated. Data were expressed as the difference from baseline values at each time point. IOP of normal rabbit eye is set to be baseline for the calculation of IOP in **Fig. 4a** (i.e. IOP mentioned as following is the difference between treated eye and normal eye). All glaucomatous eyes including control group and different pilocarpine forms injected groups had IOP around 19.9 ± 1.5 mmHg before operation. The miosis tests were carried out after acclimatization in a room with constant lighting. The pupillary diameter gauge (Smith and Newphew Pharmaceuticals, Essex, UK) was used for measuring the pupil diameter under standardized conditions. The diameters of pupil from glaucomatous eyes were taken as pretreatment baseline values. The results were presented as the average variation of pupillary diameter at each post-drug-administration time point with respect to basal levels from four independent measurements. We set the pupil diameter of normal eye as baseline and calculate the difference between each experimental group with normal eye in **Fig. 4b**.

Results (i.e. anterior chamber depth, corneal endothelial cell density, intraocular pressure and pupil diameter) were expressed as mean ± standard deviation. Comparative studies of means were performed using one-way analysis of variance (ANOVA). Significance was accepted with $P < 0.05$.

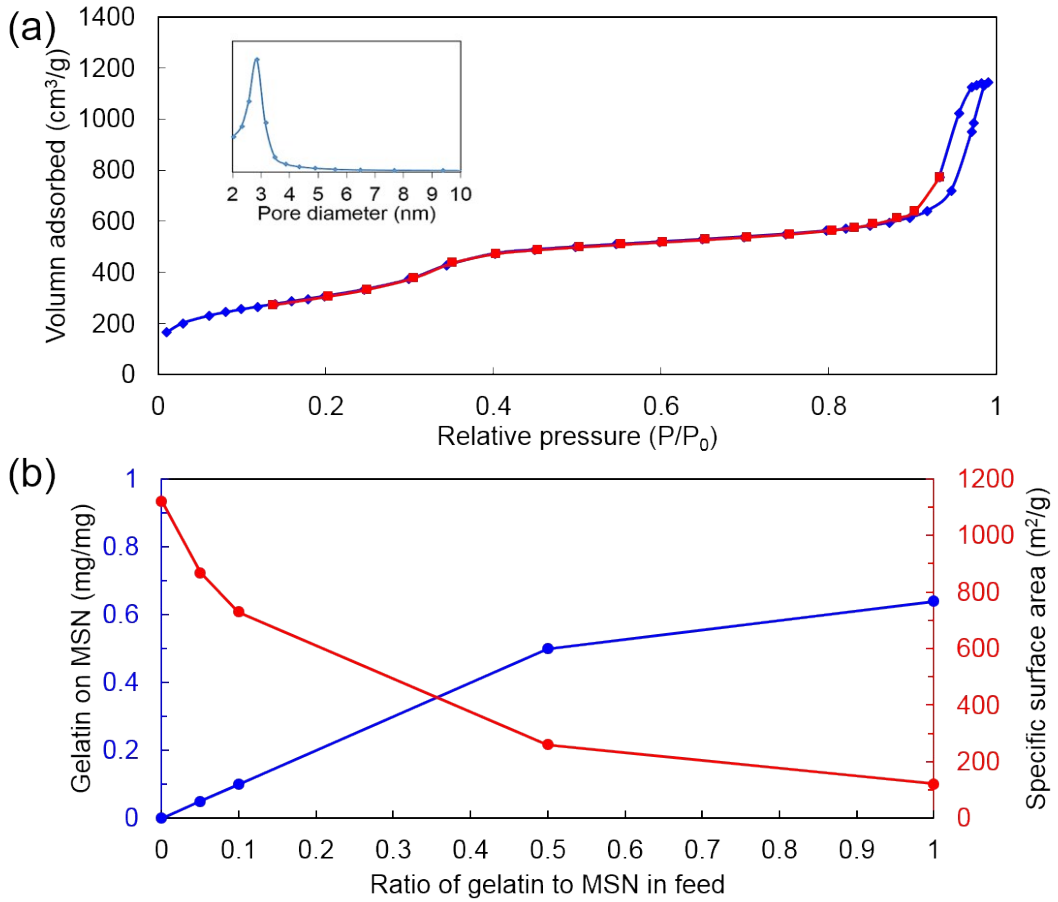

Figure S1. (a) The N_2 adsorption-desorption of MSNs and the pore size distribution of MSNs based on BJH theory. (b) The relation of gelatin on MSN and specific surface area with different ratio of gelatin to MSN in feed. The amount of gelatin on MSN was measured with thermal gravimetric analysis and the specific surface area was measured with N_2 adsorption-desorption based on BET theory.

The results showed that 0.5 was critical ratio of gelatin to MSN. The amount of gelatin on MSN was the same with the amount of gelatin in feed when the ratio of gelatin to MSN was lower than 0.5; however, the amount of gelatin on MSN became fewer as the ratio of gelatin to MSN was higher than 0.5.

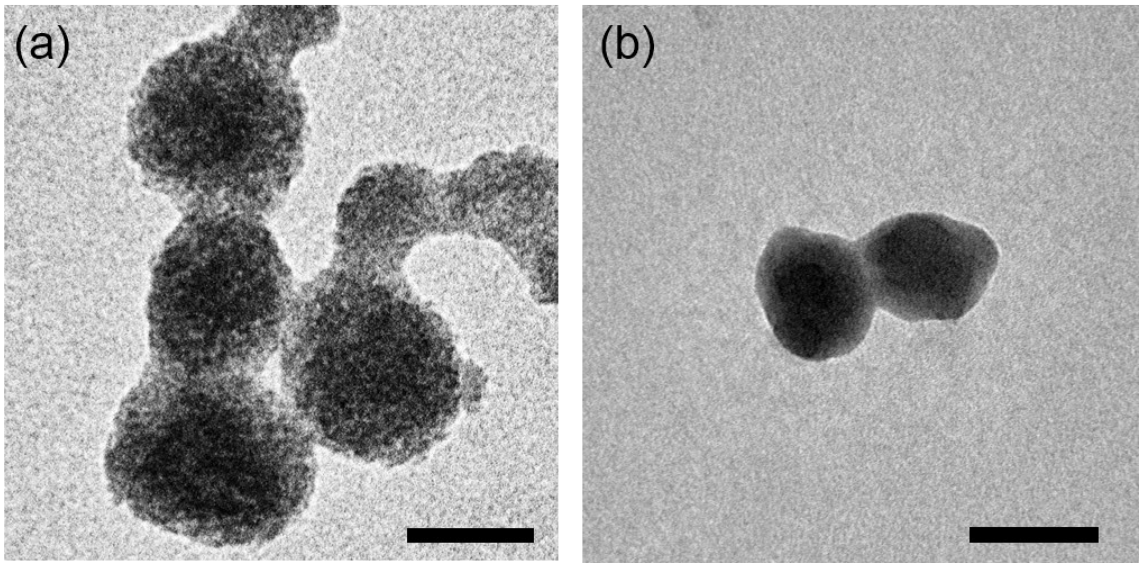

Figure S2. The TEM images of (a) GM-0.1 and (b) GM-0.5. The scalar bar is 50 nm.

Table S1. The surface area and the amount of gelatin on MSN with different amount of gelatin in feed.

Sample	Amount of gelatin on MSN (mg/mg) [a]	Specific Surface area of GM (m^2/g) [b]	Thickness of gelatin shell (nm) [c]	Zeta potential (mV) [d]	Loading capacity (μg/mg)
MSN	0	1122	0	-31.5	95
GM-0.05	0.05	868	3.21	-28.3	90
GM-0.1	0.1	730	5.66	-11.5	86
GM-0.5	0.5	261	6.65	-9.5	63

a: The amount of gelatin on MSN was measured with thermal gravimetric analysis
b: The specific surface area of GM was measured by N_2 adsorption-desorption and calculated with BET theory.
c: The thickness of gelatin shell was calculated by TEM images.
d: zeta potential was measured with Nano ZS.

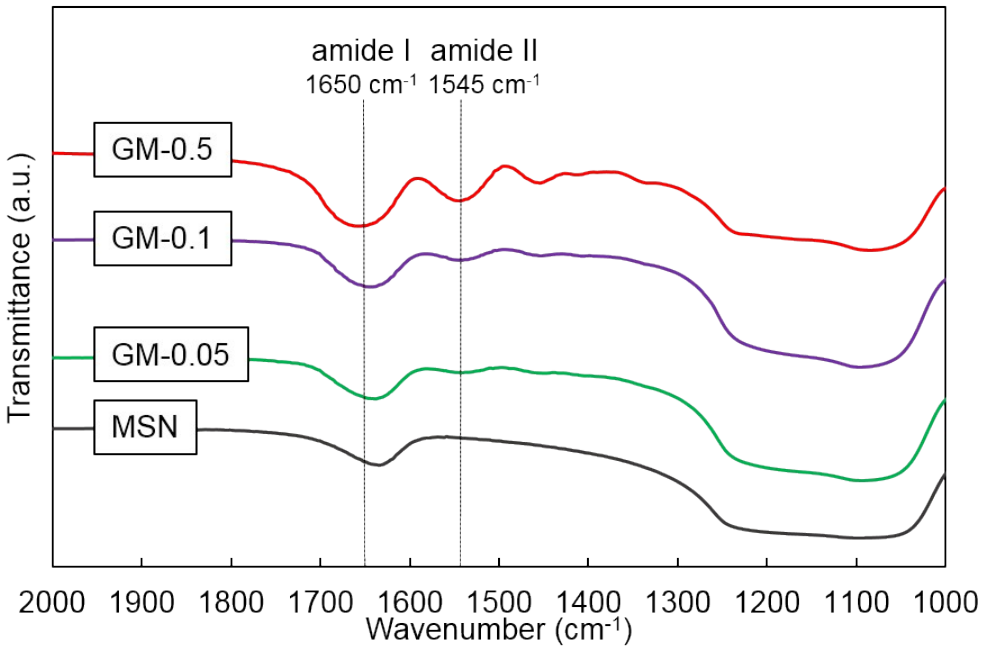

Figure S3. FTIR spectrum of MSN and GM with different amount of gelatin.

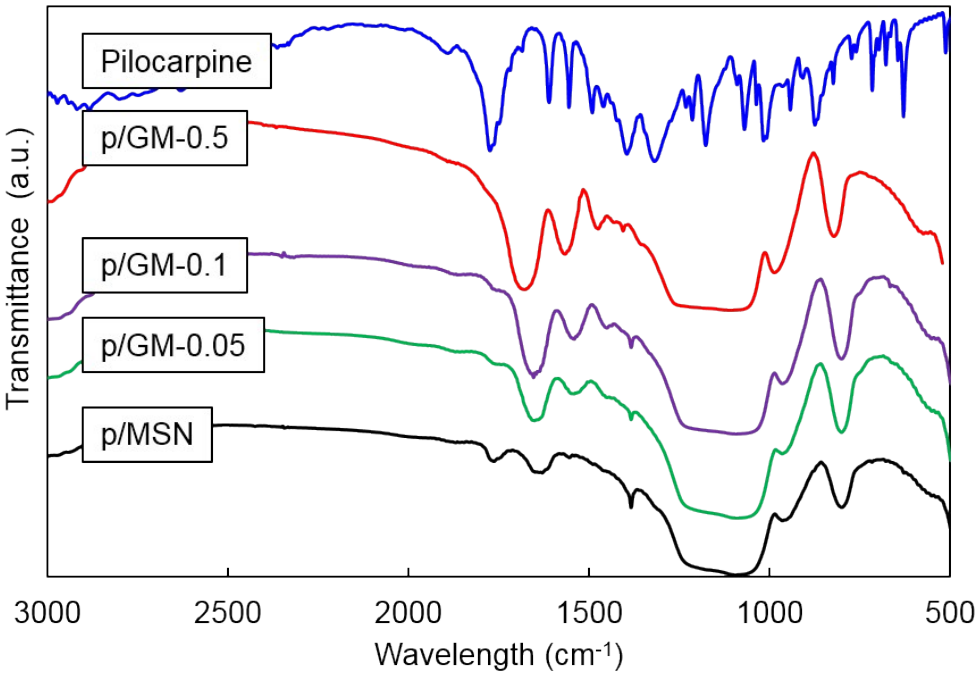

Figure S4. FTIR spectrum of pilocarpine and pilocarpine contained samples (p/MSN and p/GM-x).

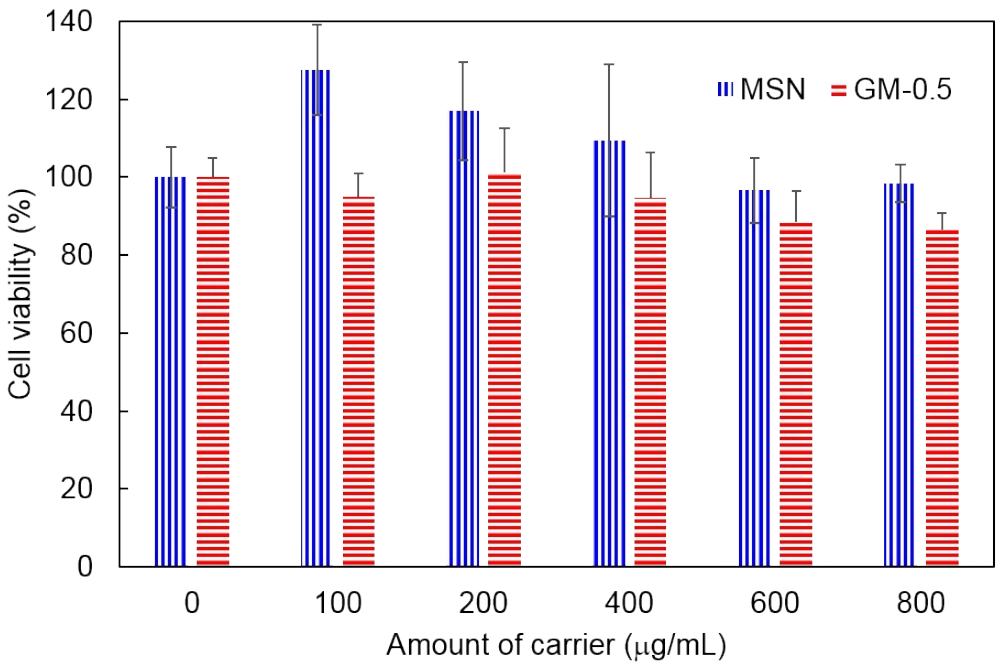

Figure S5. Cell viability of bovine corneal endothelial cells (BCE) treated with MSN and GM0.5.

As a biomaterial, the biocompatibility of material should be concerned seriously. In the anterior chamber, MSNs and GMs would interact with cells on cornea and iris. MTT assay was used to measure the biocompatibility of MSNs and GMs toward bovine corneal endothelial cells (BCE). The cell viability of BCE cell lines exposed to MSNs and GM-0.5 is shown in **Fig. S4**. The results indicated that both MSN and GM-0.5 had high biocompatibility to BCE cell lines. More than 80% of cells were alive even treated with high concentration (800 mg/mL) of MSNs and GM-0.5. MSNs have been proofed as biocompatible material and used as drug carrier in vivo. On the other hand, gelatin is a natural polymer partially hydrolyzed from collagen. Thus the combination of MSN and gelatin would not compromise the safety of GM. The well biocompatibility of GM-0.5 toward BCE cell lines indicates that it has potential to be a drug carrier for intracameral injection.

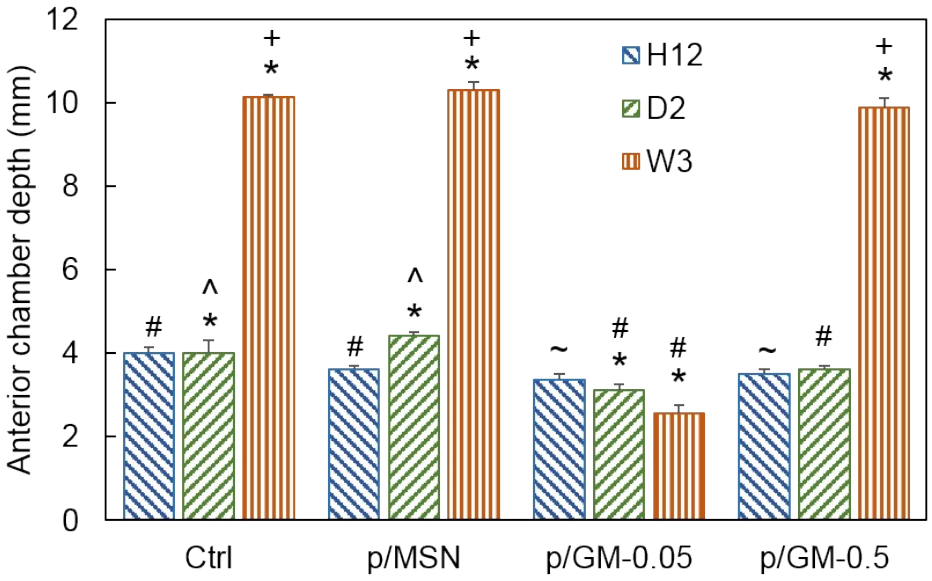

Figure S6. Quantification of anterior chamber depth 12 hours (H12), 2 days (D2) and 3 weeks (W3) after pilocarpine administration with different carriers. An asterisk indicates statistically significant differences ($^{*}P<0.05$; n=6) between two time-course in same group. A number indicates statistically significant differences ($^{\#}P<0.05$; n=6) between different groups in same time-course. A plus indicates statistically significant differences ($^{+}P<0.05$; n=6) as compared with p/GM0.05 group in same time-course. A hat indicates statistically significant differences ($^{\wedge}P<0.05$; n=6) as compared with p/GM-0.05 and p/GM-0.5 group in same time-course. A tilde indicates statistically significant differences ($^{\sim}P<0.05$; n=6) as compared with Ctrl and MSN group in same time-course.

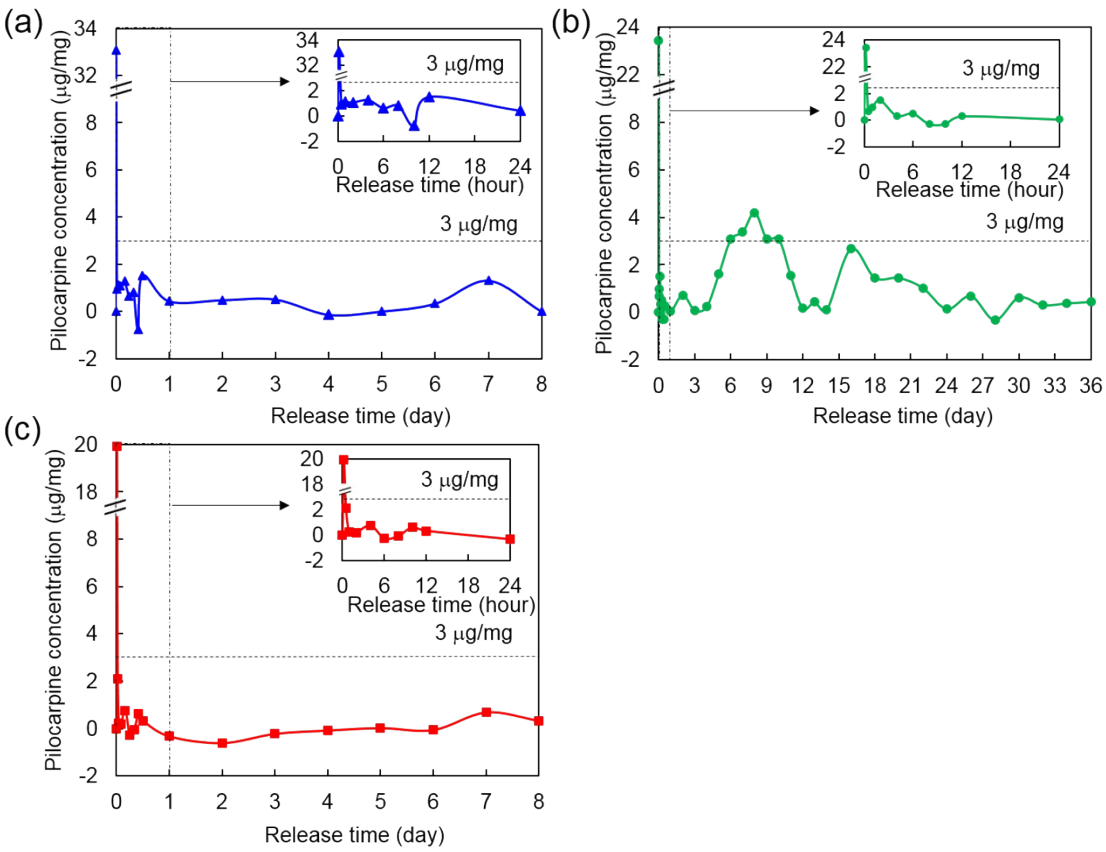

Figure S7. The profile of pilocarpine concentration releasing from (a) MSN, (b) p/GM-0.05 and (c) p/GM-0.5 in each time point.

Pilocarpine concentration in all samples decreased drastically after the burst release. The pilocarpine concentration of p/MSN decreased gradually after 12 h, and that of p/GM-0.5 also decreased gradually after 1 day. Although the pilocarpine concentration of p/GM-0.05 was close to zero after 1 day, the pilocarpine concentration increase gradually after 4 days. On the other hand, the pilocarpine concentration of GM-0.05 exceeded the minimum effective concentration of pilocarpine (3 μg/mg) to induce the contraction of iris muscle during the treatment, resulting in significant decrease of IOP after postoperative 21 days.

References

1. C. L. Percicot, C. R. Schnell, C. Debon and C. Hariton*, J. Pharmacol Toxicol Methods*, 1996, **36**, 223-228.