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Preparation of rapamycin-loaded chitosan/PLA nanoparticles for immunosuppression in corneal transplantation

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Abstract

Rapamycin-loaded chitosan/polylactic acid nanoparticles with size of about 300 nm in diameter were prepared through nanoprecipitation method using cholesterol-modified chitosan as a stabilizer. The surface coating of chitosan, which was demonstrated by zeta potential measurement, endowed the nanoparticles good retention ability at the procorneal area, facilitating the sustained release of rapamycin on the corneal. The immunosuppression in corneal transplantation of the nanoparticles was investigated using rabbit as animal model, the median survival time of the corneal allografts treated with nanoparticles was 27.2 ± 1.03 days and 50% grafts still remained surviving by the end of the observation, while the group treated with 0.5% rapamycin suspension was 23.7 ± 3.20 days. The median survival time of drug-free nanoparticles group and untreated groups were 10.9 ± 1.45 and 10.6 ± 1.26 days, respectively. The results demonstrated the excellent immunosuppression of rapamycin-loaded chitosan/polylactic acid nanoparticles in corneal transplantation.

Keywords: Chitosan; Polylactic acid; Nanoparticles; Corneal transplantation; Immunosuppression

1. Introduction

Corneal transplantation is one of the most common allografts performed. While 90% of these allografts survive and functionate satisfactorily, immune graft rejection is a major cause of graft failure. In "high risk" patients, 60% of corneal allografts can be rejected in spite of the use of topical steroids (Xie et al., 2002).

The failure in suppression of graft rejection by topical administration usually results from the drug insolubility and the difficulty to achieve clinically effective drug concentrations in the cornea and anterior chamber, even though some novel effective immunosuppressive agents, such as cyclosporine (CyA) (Milani et al., 1993) and rapamycin (RAPA) (Dong et al., 2005). Therefore, there is considerable interest in developing a delivery system which could achieve therapeutically efficacious drug concentrations localized to the anterior segment. Xie et al. have developed CyA-polylactide-co-glycolide polymer implant and tested the implant in the anterior chamber of rats with corneal allografts, demonstrating the efficacy of the local sustained release system (Xie et al., 2002). However, the most convenient method for the therapy of extraocular diseases may be topical administration since it achieves the necessary patient compliance.

The ocular efficacy of topically applied drugs is influenced by corneal contact time, the most common method to improve drug ocular availability is to increase precorneal residence time by using hydrogels based on natural, synthetic or semi-synthetic polymers (Bernatchez et al., 1993; Ludwig et al., 1992; Unlü et al., 1992). Among the polymeric carriers used, cationic chitosan (CS) has attracted considerable attention due to its unique properties such as good biocompatibility, biodegradability (Hirano et al., 1990; Knapczyk et al., 1984) and the ability to enhance the paracellular transport of drugs (Artursson et al., 1994). CS is a promising material with great application potentiality in ocular drug delivery. CS solution was in favor of prolonging the

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corneal residence time of antibiotic drugs (Felt et al., 1999). CS-coated nanocapsules were even more efficiently in enhancing the intraocular penetration of some specific drugs (Calvo et al., 1997; Genta et al., 1997). Ionically cross-linked CS nanoparticles showed prolonged residence time at the ocular mucosa after topical administration to rabbit's eyes (De Campos et al., 2001).

In previous study (Yuan et al., 2006), we synthesized cholesterol hydrophobically modified chitosan (CS-CH), and loaded the CyA into the CS-CH nanoparticles by self-aggregated method, the results demonstrated the good retention ability of the self-aggregated CS-CH nanoparticles at the procorneal area as investigated by SPECT and scintillation counter measurement. In the present work, we investigated the immunosuppressive effect of RAPA in chitosan nanoparticles for corneal transplant. For this purpose, RAPA-loaded CS/polylactic acid (PLA) nanoparticles were prepared, and the nanoparticles were topically applied to the rabbit allograft corneal in the drop formulation. The PLA was introduced into the formulation to improve the hydrophobic drug loading of the nanoparticles. The precorneal residence time of formulations was assessed by gamma scintigraphy. Grafts were examined by slit lamp microscope.

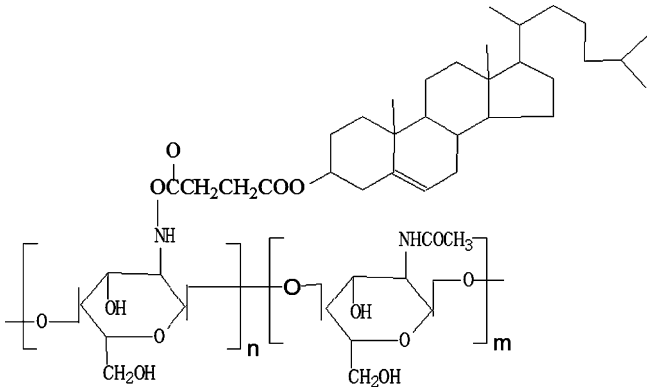
2. Experimental

2.1. Materials and animals

Chitosan samples were provided by the Nantong Suanglin Biochemical Co. Ltd. (China) with deacetylation degrees of 85% and viscosity average molecular weight of 40 kDa. Cholesterol was purchased from Tianjin Chemical Reagent Co., China, and recrystallized in ethanol before use. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) was purchased from Sigma (St. Louis, MO). Rapamycin (RAPA) was purchased from Fujian microorganism Institute (Fuzhou, China). PLA was obtained from Department of Medical Polymers Shandong Institute (Jinan, China), and the molecular weight of PLA was 10 kDa. Technetium-labeled diethylene-triamine-pentacetate (^{99m}Tc-DTPA) was purchased from Beijing Kesheng Co. The physiological saline was medical grade, and the water was purified by distillation, deionization, and reverse osmosis (MilliQ Plus). New Zealand rabbits weighing between 2.0 and 2.5 kg were obtained from the Laboratory Animal Center of Academy Military Medical Sciences, China.

2.2. Synthesis of cholesterol hydrophobically modified chitosan (CS-CH)

CS-CH was synthesized according to the previous work (Yuan et al., 2006). Briefly, cholesterol reacted with succinic anhydride to obtain cholesterol 3-hemisuccinate (CHS). The resultant CHS (0.34 mol/mol chitosan) was dissolved in *N*,*N*-dimethyl formamide (DMF), then the solution was added to a 1% (w/v) chitosan hydrochloric acid solution followed by the dropwise addition of EDC (0.18 mol/mol CHS) under stirring at room temperature. After 24 h, the reaction mixture was poured into methanol/ammonia solution (7/3, v/v). The precipitates were filtered off, washed thoroughly with methanol and deionized water. CS-CH was finally obtained by freeze-drying the precipitates. The structure of CS-CH is shown in Scheme 1. The substituted degree of CS-CH used to prepare nanoparticles was determined using elemental analysis as 1.7 cholesterol groups per 100 anhydroglucosamine units of chitosan.



Scheme 1. Chemical structure of CS-CH.

2.3. Preparation of RAPA-loaded CS/PLA nanoparticles

RAPA-loaded CS-CH nanoparticles were prepared by dialyzing the DMSO/water solution of RAPA (30 mg) and CS-CH (30 mg) against physiological saline (Yuan et al., 2006). While CS/PLA nanoparticles were prepared by nanoprecipitation method as follows: 15 mg PLA and different amount of RAPA were dissolved in 3 mL acetone, the resultant solution was then added under ultrasonication (pulse mode, 40 W, 10 min, in an ice bath at 4–7 °C) using a probe-type sonication (model 600 W, Xingzi Inc., Zhejiang, China) to 30 mL aqueous solution which contained 60 mg fresh synthesized CS-CH. The solvent was allowed to evaporate under electromagnetic stirring at room temperature for 6 h. The obtained suspension was centrifuged (16,000 × *g*, 10 min), and the collected particles were resuspended in water under ultrasonication, and then centrifuged again in order to remove the unincorporated drug. The procedure was repeated several times. Finally, the suspension was freeze-dried and the RAPA-loaded chitosan/PLA particles were obtained.

The morphology of nanoparticles was investigated by Environment scanning electron microscopy (SEM, Jeol, JSM-5600 LV). The size and size distribution of the nanoparticles were measured by dynamic light scattering (DLS) (Brookhaven 90Plus/BI-MAS instrument with BI-9000 Goniometer) with a He–Ne laser beam at a wavelength of 658 nm at 25 °C and scattering angle of 90°. Meanwhile, the ζ-potential of the particles was measured using Powereach® (Zhongchen Digital Technology Instrument Ltd., Shanghai, China). All samples were sonicated for 3 min at 40 W with probe-type sonifier for three times before each measurement.

2.4. Determination of the drug loading efficiency and in vitro drug release

In order to determine the loading efficiency of the process, 2 mg freeze-dried nanoparticles was extracted with 1 ml ace-

tonitrile (Jagdish et al., 2004). The polymer was precipitated by addition of 5 ml methanol. After centrifugation at 16,000 × *g* for 10 min, 3 ml clear supernatant was drawn out and the solvent was evaporated. The residue was then diluted with 10 ml physiological saline and the RAPA concentration was determined by UV spectrophotometer at 277 nm. The drug loading and drug entrapment efficiency were defined by the following formulas, respectively:

$$\text{Drug loading (\%, w/w)} = \frac{\text{mass of drug in nanoparticles} \times 100}{\text{mass of nanoparticles recovered}}$$

$$\text{Drug entrapment efficiency (\%)} = \frac{\text{mass of drug in nanoparticles} \times 100}{\text{mass of drug used in formulation}}$$

In vitro drug release was preformed in the physiological saline. Five milligrams RAPA-loaded nanoparticles and 1 ml saline were enclosed into a dialysis membrane (MWCO 14,000 g mol^{-1}). Then the dialysis membrane was introduced into a vial with the same saline (10 ml), and the media was stirred at 37 °C and 75 rpm. The drug release *in vitro* was last for 8 days, and at pre-determined time intervals, the entire medium was removed and replaced with the same amount of fresh saline. The amount of RAPA released from nanoparticles was determined by UV spectrophotometer.

2.5. Radiolabeling and ocular distribution of nanoparticles

The radiolabeling of CS-CH/PLA nanoparticles was performed according to the method reported by Banerjee et al. (2002). Lyophilized nanoparticles (2 mg/ml) were dispersed into Tris–HCl buffer solution (pH 7.2). Nitrogen purging, prior to mixing was carried out to degas all solutions. To 0.1 ml ^{99m}Tc (5.8 mCi) in saline, 5 mg of solid sodium borohydride was added directly with continuous stirring followed by immediate addition of the nanoparticles buffer. The solution was stirred for 20 min at room temperature, then centrifugated at 18,000 rpm for 10 min followed by washing three times with buffer under sonication condition. To make a control, RAPA was firstly dissolved using a little ethanol and then dispersed saline, and the suspension was radiolabeled by addition of 1.6 mCi ^{99m}Tc-DTPA solution.

The ocular distribution of nanoparticles was assessed by scintillation counter and single photon emission computed tomography (SPECT, GE DISCOVERY-VH, USA) images analysis. After installation of 25 μl radiolabeled nanoparticles solution or RAPA suspension onto the left cornea of rabbit, ocular dynamic SPECT imaging was immediately initiated. A small plastic vial containing 0.5 ml radiolabeled CS-CH solution was placed near to lachrymal duct as an orientation tracer (Fig. 3a). The SPECT images were collected at defined time interval.

The rabbits were sacrificed after SPECT measurement. Immediately after sacrificed blood samples were collected from the marginal ear vein. The eyes were extirpated, and the remaining radioactivity was counted with scintillation counter. Then aqueous humour was withdrawn from the anterior chamber, and cornea, conjunctiva and iris/ciliary body were subsequently dissected. Each tissue, except cornea and conjunctiva, was rinsed with physiological saline. The radioactivity of all samples was recorded.

2.6. Corneal transplantation and immunosuppression

2.6.1. Corneal transplantation

Three interrupted 5-0 silk sutures were placed in the anterior cornea of the recipient right eyes and vascularization was induced in 10–12 days. When the blood vessels had grown into the recipient cornea, the sutures were removed and a donor cornea was transplanted into the site. The rabbits were randomly divided into four groups with 10 rabbits for each. In the first group (RAPA group), the grafted eye received 0.5% drops. In the second group (RAPA-particles group), the recipient eyes received CS-CH/PLA nanoparticles drops which contain equivalent RAPA with that in RAPA group. While in the third group (particles group), the grafted eye received empty CS-CH/PLA nanoparticles drops. Both groups were treated two times a day for 4 weeks following surgery. The last group was the control group, the grafted eye was not treated throughout the observation period.

Recipients of corneal allografts were examined every 3 days for 4 weeks by a blinded observer using a slit lamp microscope. Clinical appearance of each graft was scored using the following three criteria: graft opacity, graft edema, and graft neovascularization. The analysis of which was recorded as rejection index (RI). Rejection was considered only when RI ≥ 5. The criteria for the allografts scoring was as follows (Holland EJ et al., 1991): (A) Graft opacity: 0, no opacity; 1, slight opacity—details of iris clearly visible; 2, some details of iris no longer visible; 3, pronounced opacity while pupil still recognizable; 4, total opacity. (B) Graft oedema: 0, no oedema; 1, mild oedema; 2, moderate oedema with raised transplant; 3, heavy oedema with blisters visible on the surface of the allografts. (C) Graft neovascularization: 0, no vessels entering the edge of the allografts; 1, vessels entering the peripheral zone of the allografts; 2, vessels entering the mid-peripheral zone of the allografts; 3, vessels entering the central zone of the allografts.

2.6.2. Statistical analysis

The survival time and the clinical scoring data were compared between the various groups by means of one-way ANOVA on computer (SPSS for Windows). All data were expressed as mean ± standard deviation (S.D.). A $P < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Preparation of CS-CH/PLA nanoparticles

The chemical structure of CS-CH was shown in Scheme 1. The compound possesses amphiphilic property owing to the hydrophobically modification of chitosan by cholesterol, thus can self-aggregate into nanoparticles with hydrophobic microenvironment inside. Meanwhile, CS-CH possesses surface activity and can also be used as stabilizer for the preparation of spheres

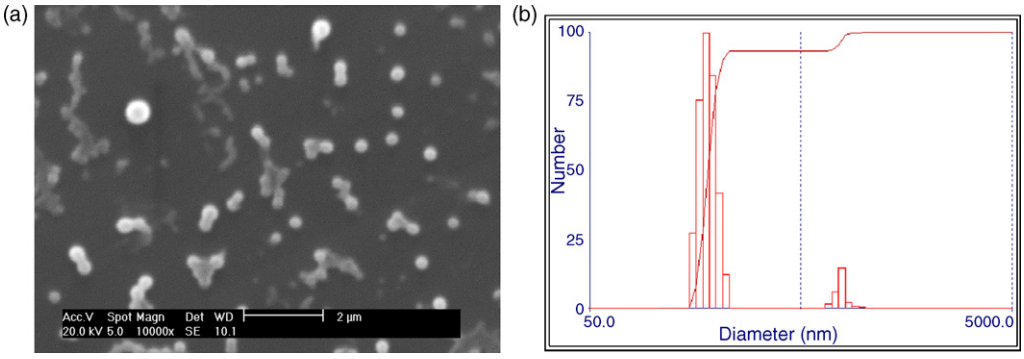


Fig. 1. SEM micrographs and size distribution of CS-CH self-aggregates (a) SEM micrographs. (b) Size distribution measured by DLS.

composed of hydrophobic polymer. When 15 mg PLA was added to the aqueous solution of CS-CH under ultrasonication, no precipitate was found but the solution changed from optical clear to slightly turbid, indicating the increase in size of the obtained particles. This phenomenon was consistent with the SEM observation and DLS measurement. As shown in Fig. 1 and Table 1, the addition of PLA slightly increased the size of the CS-CH nanoparticles, and meanwhile, broadened the size distribution of the particles. A further increase of PLA led to an abrupt increase in size of the particles from nanometer to micrometer level. These large particles will increase patient discomfort.

The zeta-potential of the CS-CH and CS-CH/PLA nanoparticles was measured using Powereach® zeta potential measuring instrument. The CS-CH/PLA nanoparticles exhibited a positive charge zeta potential as 30.3 mV, which was close to that of CS-CH nanoparticles (44.6 mV), suggesting that the PLA was packed in the nanoparticles since its zeta potential was −35.2 mV.

3.2. Drug loading and in vitro drug release

In our previous study, we prepared CyA-loaded CS-CH nanoparticles by dialysis method. The effort to enhance the loading of RAPA in CS-CH nanoparticles had unremarkable effect in the present work. As shown in Table 1, the maximum drug content was lower than 5.6% even when the theoretical loading efficiency reached 50%.

The addition of PLA significantly improved the drug loading efficiency of the nanoparticles due to the stronger hydrophobic interaction between RAPA and PLA. The actual drug loading efficiency increased with the concentration of RAPA, and finally reached the equilibrium at 18–29%.

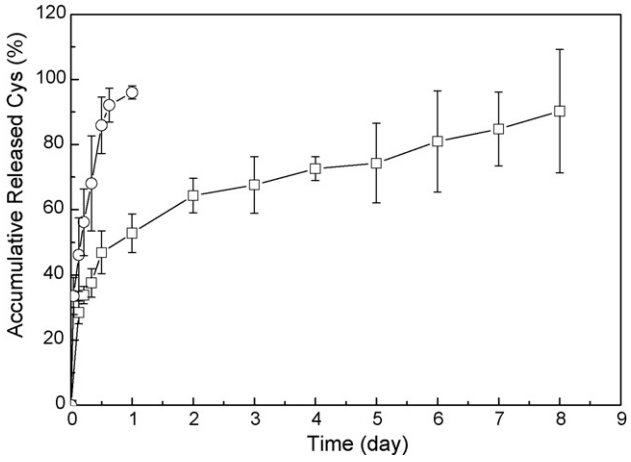


Fig. 2. Profile of RAPA release from CS-CH nanoparticles (□) and CS-CH/PLA nanoparticles (◯). (The drug-loading efficiency of the CS-CH/PLA nanoparticles was 25.1%. The release was performed *in vitro* in physiological saline at 37 °C.)

The incorporation of PLA altered the releasing behavior of the drug from particles. Fig. 2 showed the release profiles of RAPA from both CH-CS/PLA nanoparticles and CS-CH nanoparticles. As illustrated in the plot, the incorporation of PLA into CS-CH particles leads to a much slower sustained release of RAPA. After 12 h, approximately 50% of RAPA is released from CS-CH/PLA nanoparticles as compared to 85% from CS-CH samples. This prolonged release is attributed to the hydrophobicity of PLA. When PLA was introduced into CS-CH particles, RAPA prefers to disperse in PLA microenvironment. The hydrophobicity of PLA restricts the permeation of water into the particles, preventing the diffusion of RAPA out of particles. The initial fast release is attributed to RAPA adsorbed on the nanoparticle surface (Magenheim et al., 1993)

Table 1
Properties of RAPA-loaded CS-CH/PLA particles

Sample	RAPA (mg)	Mean diameter[a] (nm)	Variance[a]	Loading efficiency (%)	Encapsulation efficiency (%)
CS-CH	30	219	0.134	3.2 ± 2.4	6.4 ± 4.8
CS-CH/PLA	10	326	0.225	18.2 ± 0.8	89.3 ± 9.4
CS-CH/PLA	15	317	0.218	22.4 ± 1.3	83.9 ± 6.5
CS-CH/PLA	20	312	0.284	29.1 ± 1.5	75.2 ± 11.3

[a] Effective diameter and variance determined by dynamic light scattering.

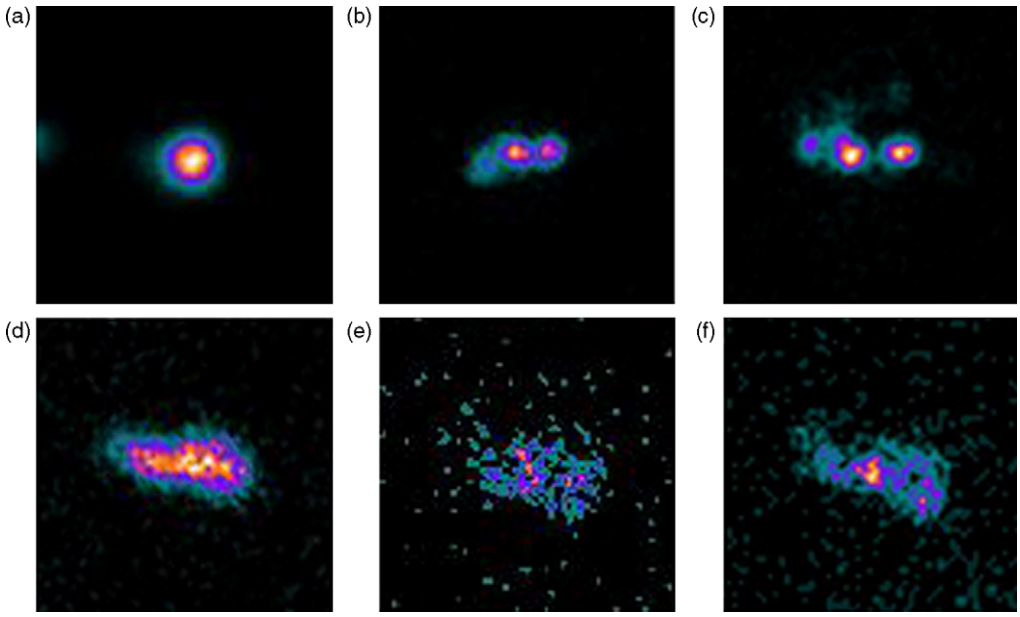


Fig. 3. Ocular distributions of CS-CH/PLA and RAPA suspension observed by SPECT. The images were recorded for CS-CH/PLA nanoparticles at (a) 2 min, (b) 65 min, (c) 127 min; and RAPA suspension at (d) 2 min, (e) 70 min, and (f) 106 min.

and/or to the release of the drug encapsulated near to nanosphere surface. Followed by the initial stage, a constant slow RAPA release was observed within 8 days until 90% of the loaded drug amount, showing a typical sustained and prolonged drug release which depends on drug diffusion and matrix erosion mechanisms (Holland and Tighe, 1992; Musumeci et al., 2006).

3.3. The ocular distribution

The ocular distribution of CS-CH/PLA nanoparticles was evaluated by SPECT image analysis and scintillation counter. The observation of the acquired gamma-camera images was shown in Fig. 3. As a control sample RAPA suspension showed a good spreading over the entire precorneal area immediately after the topical administration (Fig. 3d). Most of these eye drops drained into lachrymal duct, and got into the lacrimal sac, resulting in rapid reduction in the radioactivity.

The CS-CH/PLA nanoparticles congregated at conjunctival sac, which probably due to the high viscosity of chitosan solution (Fig. 3a). Only a little part of this applied dose was drained into lacrimal sac at 65 min post-administration, while the major fraction of the instilled dose remained on the ocular surface where they separated into two parts retaining on the inner and outer canthus, respectively. No obvious decrease in radioactivity was observed till the end of the measurement.

The rabbits were sacrificed after SPECT measurement, and the remaining radioactivities of the two formulations were counted with scintillation counter. The remaining activities were 67.3% and 11.6% for CS-CH/PLA nanoparticles and RAPA suspension, respectively. These results were well consistent with the observation of SPECT.

The remaining activity in the cornea, conjunctiva, iris/ciliary, aqueous humour and blood after topical administration of the two formulations for about 150 min was shown in Fig. 4. The animals treated with CS-CH/PLA nanoparticles showed significantly higher remaining radioactivities on corneal and conjunctival ($P < 0.05$) than those treated with a suspension of RAPA (two to six times increase). In general, after topical administration of a conventional ophthalmic drug solution, the contact time of drug with ocular tissues was relatively short (1–2 min) because of the permanent production of lacrymal fluid (0.5–2.2 ml/min) and drainage of lacrymal fluid during blinking (every 12 s) towards the nasolacrimal duct (Ahmed and Patton, 1985, 1987). A major fraction of the instilled dose was thus absorbed systemically by this drainage (Lang, 1995), and part of them reached the bloodstream (Chang and Lee, 1987). This is the reason why the RAPA suspension exhibited a higher remaining activity in blood as shown in Fig. 4. When CS was introduced

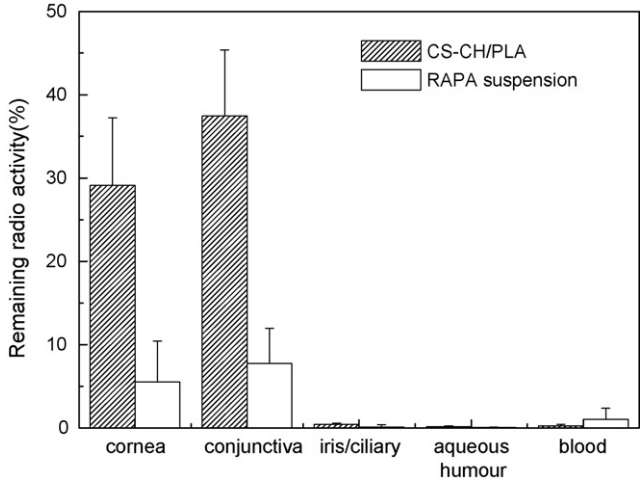


Fig. 4. Remaining radioactivity of CS-CH/PLA nanoparticles and control formulations consisting of a RAPA suspension aqueous solution in the ocular tissues and blood after topical administration into rabbits.

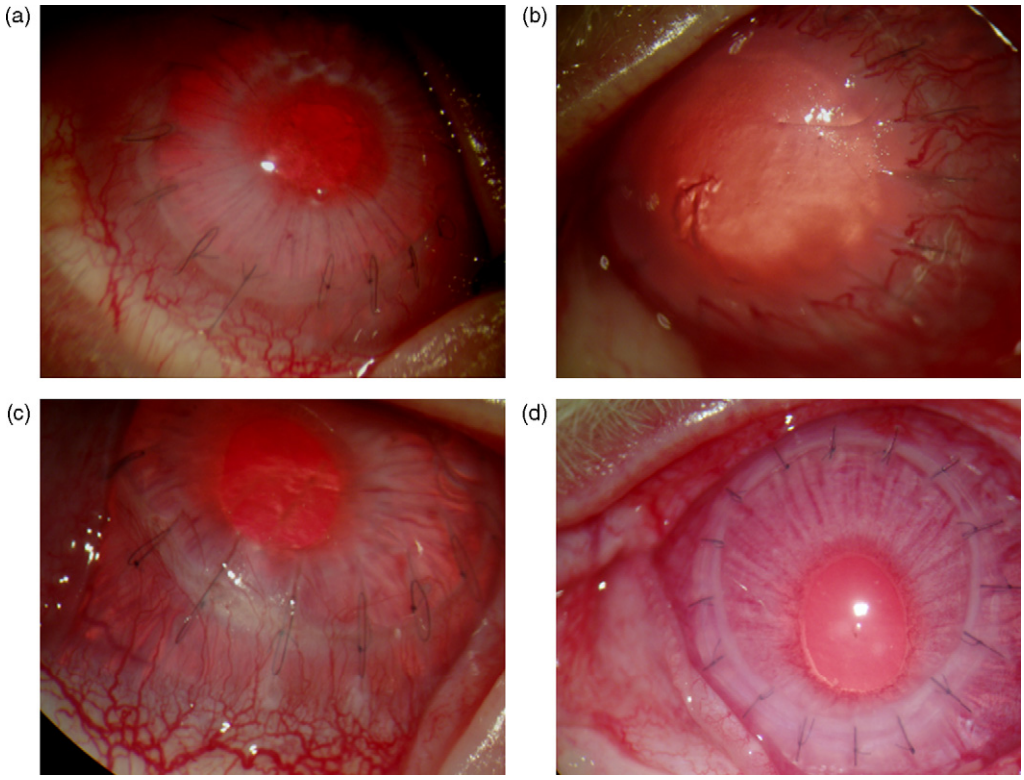


Fig. 5. Recipients of corneal allografts observed by slit lamp microscope 10 days after transplantation. (a) Control group, (b) empty particles group, (c) RAPA group, and (d) RAPA-particles group.

into the drug formulation, the residence of drug on the ocular surface was prolonged due to the mucoadhesive character of CS mediated by the electrostatic interaction between the positively charged CS and the negatively charged corneal and conjunctival cell (He et al., 1998; Henriksen et al., 1996; Lehr et al., 1992). Indeed, it was found that CS in the form of nanoparticles plays a key role in improving their interaction with the ocular surface, and it was also observed that fluorescence-labeled nanoparticles remain attached to the cornea and the conjunctiva for at least 24 h. This mucoadhesive character of CS nanoparticles will facilitate the drug delivery to ocular surface to cure the extraocular diseases, such as keratocon junctivitis sicca, dry eye disease and immune rejection after corneal transplantation.

Fig. 4 also showed the remaining radioactivity in the aqueous humour and iris/ciliary body, respectively. The radio levels in aqueous humour and iris/ciliary body were quite low, which near to that of background. And no statistical differences ($P > 0.05$) were observed in the remaining activity attained in these intraocular tissues after topical administration of the two formulations. The blood-cornea-barrier not only hindered the transport of the small molecule drug, but also the macromolecules nanoparticles due to the complex structure of cornea.

It should be noted that the results showed here only represent the distribution of drug carrier in the eyes for the CS-CH/PLA formulation, not the RAPA that loaded in the nanoparticles. The study on drug distribution was hindered by the difficulty in radiolabeling of RAPA. But the prolonged residence of nanoparticles on the ocular surface will facilitate the drug absorption by the external ocular tissues as proved by De Campos et al. (2001).

3.4. Immunosuppressive effect of RAPA-loaded CS-CH/PLA nanoparticles

In all groups we studied, mild corneal edema was observed immediately after surgery. The edema disappeared in 3 days after transplantation. Enlarged blood vessels were observed at the graft–host junction, particularly around the sutures. Five to seven days after surgery, some of these vessels began to enter the grafts. After treated with RAPA suspension or RAPA-loaded nanoparticles, the vessels disappeared gradually and the corneal were clear. In contrast, the corneals without treatment or treated with empty nanoparticles were opaque, and severe stromal edema and neovascularization could be found within 28 days. (Fig. 5)

All of the 10 grafts in the untreated control group were rejected within 13 days. The median survival time of these grafts was 10.6 ± 1.26 days. Animals in the empty nanoparticles group rejected the corneal allografts in a median time of 10.9 ± 1.45 days and none of these grafts survived beyond 13 days. In the RAPA suspension group, grafts were rejected between 19 and 27 days with a median survival time of 23.7 ± 3.20 days. In the RAPA-loaded nanoparticles group, the median survival time of the grafts was 27.2 ± 1.03 days and 50% grafts still remained surviving by the end of the observation (Fig. 6).

Statistically, there was no significant difference in the median survival time of the grafts between the control and empty nanoparticles groups (Q test analysis revealed that $P > 0.05$.) Both RAPA suspension and RAPA-loaded nanoparticles groups

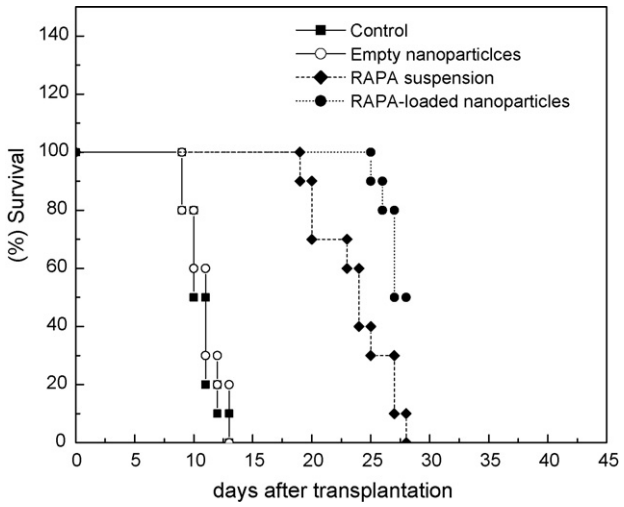


Fig. 6. Survival curves of corneal allografts in rabbits treated with empty particles, RAPA suspension and RAPA-particles.

had statistical significant difference in the median survival time compared with the control and empty nanoparticles groups ($P < 0.01$). And there was a statistically significant difference between the RAPA suspension and RAPA-loaded nanoparticles groups ($P < 0.01$).

It should be noted that the nanoparticles were administered twice a day, this may not be the optimum period for this formula application. The optimum parameters, such as the dose and the interval of formula applying, the release period of formula which was related to molecular weight of PLA, will be further investigated.

4. Conclusions

RAPA-loaded chitosan/PLA nanoparticles were prepared through nanoprecipitation method using cholesterol-modified chitosan as stabilizer. The nanoparticles were radiolabeled by ^{99m}Tc, and their ocular distribution was studied by SPECT and scintillation counter. Chitosan/PLA nanoparticles showed better retention ability at the procorneal area compared with RAPA aqueous suspension. Taking advantage of this property, the RAPA-loaded chitosan/PLA nanoparticles were used to treat corneal allografts, and the results indicated that the nanoparticles showed an excellent immunosuppressive effect compared with the RAPA eye drops.

Acknowledgements

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