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Preparation and properties of a drug release membrane of mitomycin C with *N*-succinyl-hydroxyethyl chitosan

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Abstract A novel drug loaded membrane made of *N*-succinyl-hydroxyethyl chitosan and mitomycin C was used as an implant for glaucoma filtering surgery. The characteristics of the membrane, such as FTIR, equilibrium water content, swelling ratio, permeability, and drug release in vitro were determined. The L929 fibroblast inhibition of drug loaded membranes was compared to hydroxyethyl chitosan film and blank control, detecting by MTT. The biodegradability and biocompatibility were evaluated by implanting membranes into the subcutaneous tissue and muscle of rats. FTIR indicated mitomycin C was introduced. The experimental results indicated the drug loaded membrane was effective on the swelling property, permeability, and drug release in vitro. Cell culture experimental results demonstrated that the destination membrane inhibited fibroblast proliferation. In vivo, the membranes showed bioabsorption and biocompatibility. The experimental results provide a theoretical basis for the future development of the drug loaded membrane as an implant for increasing the success rate of filtering surgery.

1 Introduction

Glaucoma is second to cataract as the leading cause of irreversible blindness worldwide [1, 2]. Early diagnosis and effective treatment may remarkably decrease the rate of blindness in glaucoma patients [3]. Reducing the intraocular pressure (IOP) is the main method to treat glaucoma in the clinic. Trabeculectomy is first described by Cairns in 1968 [4] and becomes the most-performed glaucoma surgery still used today. It is a well-studied and established method of surgically lowering IOP by creating a drainage bypass from the anterior chamber to the subconjunctival space with filtering bleb formation. Although techniques and the adjunctive use of antimetabolites has enhanced long-term success as measured by IOP control, trabeculectomy remains a high-risk surgical operation with the risks including blebitis and endophthalmitis, over filtration and hypotony, corneal endothelial cell loss, dellen, bleb overhang, bleb leaks, bleb fibrosis and encapsulation, and aqueous misdirection [5].

The loss of the IOP-lowering effect may be caused by collapsed of filtering bleb. Its formation is referable to scar formation between the conjunctiva and sclera. Scarring of the filtering bleb is associated with cellular proliferation, particularly fibroblast proliferation [6, 7]. Antimetabolite is used for inhibition of fibroblast proliferation during an operation or after, which increases the success rate of filtering surgery [8]. Mitomycin C (MMC) in particular is widely used as an antifibrotic agent to inhibit fibroblast proliferation in trabeculectomy [9]. MMC has higher activity under hypoxia and will effectively prevent bleb fibrosis if be used prior to angiogenesis in the wound [10]. MMC inhibits scarring chemically, but the chemical reaction might cause unexpected complications and tissue damage. The risk of wound leakage, ocular hypotony, hypotonous maculopathy, blebitis, and bleb-related endophthalmitis is increased [11]. An antifibrotic that is harmless to the eyes is desirable, which to maintain the filtering bleb without or with fewer complications caused by conventional antifibrotic agents. It is reported that the MMC application in low-concentration to the episclera

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during trabeculectomy can inhibit cicatrization at the bleb site [12, 13].

Biomembranes that can be used in glaucoma filtering surgery have developed rapidly. Materials made up of distinct components have been implanted in vitro and in animal models to evaluate the capability for reducing postoperative adhesion between the conjunctiva and sclera [14–16]. These biomembranes show the performance of antifibrotic because they create not a chemical but a physical barrier between the conjunctiva and sclera. An agent used for trabeculectomy in human eyes should be safe, biodegradable, and easy to apply, and it should have an IOP-lowering effect. Chitosan (CTS) is a new type of biological material with low immunogenicity [17], biodegradability, non-toxic, no harmful degradation products [18, 19], and antibacterial [20]. It can inhibit the growth of fibroblasts [21]. In addition, CTS has good mechanical properties and cationic characteristic [17, 19]. Therefore, CTS and its derivatives draw attention as a drug delivery vehicle [17, 22, 23].

In order to improve the hydrophilicity and biocompatibility of CTS, a water-soluble derivative of CTS, hydroxyethyl chitosan (HECTS) has been prepared. Our previous study showed that HECTS had good solubility in water, no cytotoxicity, antibacterial property, and wound healing activity. The membrane of HECTS can be easily applied as an implant agent by cutting it into suitable sizes for trabeculectomy. In order to obtain free carboxyl group, *N*-succinyl-HECTS (Suc-HECTS) is prepared by HECTS and succinic anhydride via ring-opening reactions. Due to the existence of $-NH_2$ and –COOH groups in its structure, Suc-HECTS shows good hydrophilicity and can easily react with many kinds of agents [24, 25]. It is valuable for the drug-carrier to readily prepare its conjugates with various drugs to avoid vexatious complications [26, 27]. This review deals with the application of Suc-HECTS as a carrier of water-soluble drug conjugate in chemistry.

Theoretically, a chemical combination could happen between MMC and Suc-HECTS in the presence of appropriate catalyst. Then the membrane of Suc-HECTS-MMC may be prepared by cross-linked modification. The ideal membrane should meet the above-mentioned requirements and may be a suitable agent for inhibiting fibrosis and preventing postoperative adhesion after trabeculectomy.

In this study, we designed the synthesis technology of Suc-HECTS by introduction of succinyl groups into N-terminal of the glucosamine units. The preparation and properties of a novel drug loaded membrane made of Suc-HECTS and MMC were described. We studied the inhibition of the drug loaded membrane on cell attachment and growth by culturing the L929 fibroblast. In addition, the biodegradability and biocompatibility of the membrane were evaluated in vivo.

2 Materials and methods

2.1 Materials and regents

Wistar rats were purchased from Qingdao Laboratory Animal Center (female, weight 200 g). HECTS (degree of deacetylation = 95%; Mw = 130 kDa) was prepared and purified in our lab. Biodegradable cross linker (1,4-Butanediol diglycidyl ether, BDDGE) and diphenyl tetrazolium bromide (MTT) were purchased from Sigma Chemical Co.(USA). Materials for cell culture including Dulbecco's Modified Eagle Medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco Co. (USA). Tissue culture flasks and 48-well plates were obtained from Corning Co. (USA). MMC for injection was purchased from Zhejiang Hisun Pharmaceutical Co Ltd. (Taizhou, China). All other reagents were obtained commercially as reagent-grade products.

2.2 Preparation and characterization

2.2.1 Synthesis and characterization of Suc-HECTS

Hydroxyethyl chitosan (2 g) was dissolved in deionized water (50 ml), then a solution of succinic anhydride (9.76 g) in dimethyl sulphoxide (10 ml) was added and stirred at 40°C. After stirring for 24 h, the conjugate was separated and purified by ultrafiltration with a molecular weight cut-off limit of 80,00–14,000 at 4°C for 3 days. Finally, the compound was obtained as a floccus by lyophilization.

The product was characterized by FTIR and ^{1}H NMR spectra. IR spectra of HECTS and Suc-HECTS were obtained with a FTIR spectrophotometer (Nicolet Nexus 470, NICOLET, USA) in the 4,000–400 cm^{-1} range. The ^{1}H NMR spectra were recorded for solution in D_2O on spectrometer (Jeol JNM-ECP 600, JEOL, JAPAN) with tetramethylsilane (Me_4Si) as the internal standard, and chemical shifts were recorded in values. Degree of succinyl substitution (DS) was determined from the area ratio of substituted group protons and methyl proton of *N*-acetyl group.

2.2.2 Preparation of Suc-HECTS–MMC membranes

A weighed amount of Suc-HECTS was added into rapidly stirred distilled water (4 ml). The desired amount of MMC was added and completely dissolved. Then a solution of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride(EDC·HCl) in distilled water was added dropwise. After stirring for 30 min, a suitable amount of cross linker was added and the solution was stirred for 45–60 min. All the above operations were carried out at room temperature and avoided light. The whole synthesis route is shown in Fig. 1.

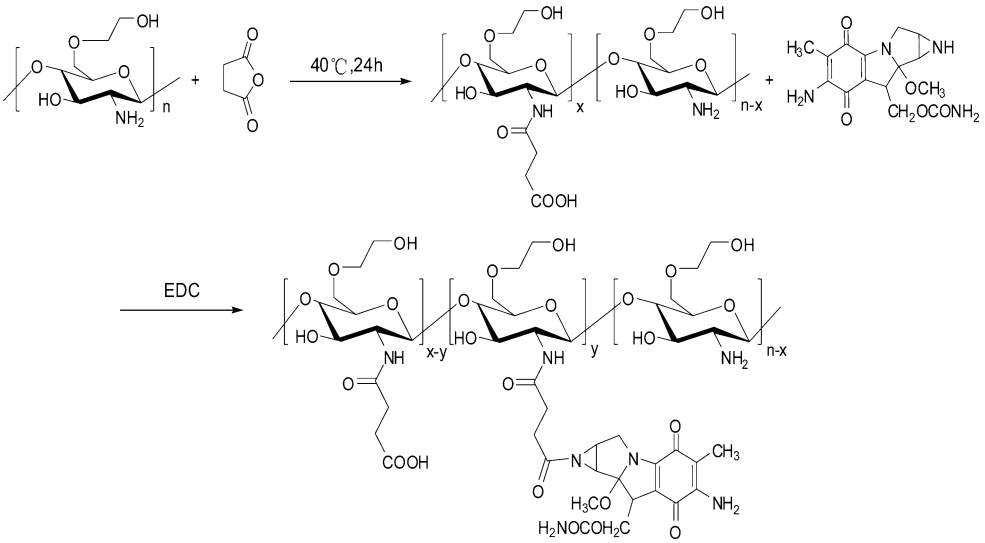

Fig. 1 The synthesis of Suc-HECTS–MMC

In addition, a blank control film was devised: HECTS was dissolved in deionized water (4 ml). Then a suitable amount of cross linker was added. The reaction solution was stirred for 2 h.

After cross-linking reaction, the resultant solutions were poured into spotless square glass sheets of 4 cm side which were balanced by using the leveling feet. The contents in glass sheets were laid to dry to form thin membranes.

Membranes for in vitro cell culture and in vivo implantation studies were prepared under sterile conditions in a biosafety level II cabinet. All regents were either autoclaved or sterilized by filtering with 0.2 μm filters.

The relationship between cross-linking degree and MMC release in vitro was investigated through single factor design experiment. Three cross-linking degrees were designed in this study: 20:1, 50:1, and 100:1(*n*/*n*), based on the mole number of Suc-HECTS. According to Table 1, four kinds of membranes were prepared, respectively, and recorded as 0#, 1#, 2#, and 3#. 0# membrane without drug was used as the blank group in subsequent experiment.

Table 1 Experimental design for relationship between cross-linking degree and MMC release in vitro

Sample	Material (Weight in mg)	MMC (Weight in mg)	Degree of cross-linking (*n*[CTS]: *n*[cross linker])
0#	HECTS (250)	0	50:1
1#	Suc-HECTS (250)	2	20:1
2#	Suc-HECTS (250)	2	50:1
3#	Suc-HECTS (250)	2	100:1

2.3 Characterization and determination of Suc-HECTS–MMC membranes

2.3.1 FTIR

The Infrared (IR) spectrum of the membrane of Suc-HECTS–MMC was recorded with a FTIR spectrophotometer (Nicolet Nexus 470, NICOLET, USA) by attenuated total reflection (ATR) techniques. The sample was scanned in the range of 4,000–400 cm^{-1}.

2.3.2 Measurement of swelling property

The equilibrium water content of membrane was defined as the weight ratio of water content to the swollen membrane, and the swelling ratio was defined as the volume change ratio of membranes between the dried and the swollen. Dried membranes were initially cut into about 10 mm length square films and measured their thickness (δ1), length (d1), and weight (w1). They were immersed in 0.1 M phosphate buffered saline (pH 7.4) for 24 h at 37°C until the membranes adsorbed water to equilibration. Then the membranes were removed from the buffer solution, placed between two pieces of dried filter paper to remove excess solution, and then measured again (noted as δ2, d2, and w2).The percentage of equilibrium water content (%) and swelling ratio were calculated with the following equations:

Equilibrium water content (%) = [(w2 − w1)/w2] × 100%

Swelling ratio = $d2^2\delta2/d1^2\delta1$

Five specimens were measured for each membrane to obtain an averaged value.

2.3.3 Measurement of permeability

The 1#, 2#, 3#, and 0# membranes (15 mm in diameter) were placed into permeability apparatus without leaking separately. The apparatus was divided into two areas which were not connected by membranes. One side was added to

the design capacity of the infiltration solution (0.9% NaCl, 1% glucose, and 0.4 mg/ml tyrosine) and the other side was deionized water. Then the apparatus was placed into an incubator at 37°C and 100 rpm. The ion concentrations of solutions on both sides were periodically measured by specifically detection means. Conductimetric method, 3, 5-Dinitrosalicylic acid (DNS) method [28] and ultraviolet spectrophotometry were used to determine the content of NaCl, glucose, and tyrosine in solutions.

The experiment was repeated three times.

2.3.4 Determination of the drug release in vitro

Experiments for the drug release in vitro from the drug loaded membranes (1#, 2#, 3#; 1 × 1 cm) were performed at 37°C in 0.1 M PBS, pH 7.4. The release medium was collected in regular time [27, 29]. The volume and concentration of PBS were maintained by replacement with fresh buffer each time. This procedure was repeated for 360 h. A colorimetric procedure, previously established, was used for MMC quantification. The method is based on ultraviolet spectrophotometry. The absorption of the MMC solution at 364 nm had a linear relationship with the MMC concentration. The different concentration gradient solutions of MMC were prepared and their ultraviolet absorbancies at 364 nm were determined. Then the standard curve of the relationship between MMC concentration and $OD_{364\ nm}$ was confirmed. The content of MMC in releasing solution was taken from the standard curve.

The experiment was repeated three times.

2.4 L929 fibroblast culture on membranes

In this study, L929 fibroblastic was used for the experiments of cell proliferation. L929 fibroblastic cells were revived using DMEM supplemented with 10% FBS, and seeded on 25 cm^2 culture flasks. They were incubated at 37°C in an atmosphere containing 5% CO_2 with the culture medium changed every 2 days. After reaching 80% confluence, the fibroblast cells were rinsed third with D-Hanks' balanced salt solution, incubated with a mixture of 0.25% trypsin and 0.02% EDTA at 37°C for 5 min, then neutralized with a culture medium containing 10% FBS. The cells at a density of 5.0×10^5 cells/ml were cultured on 25 cm^2 plastic flasks in a CO_2 (5%) incubator at 37°C.

Sterile 0#, 1#, 2#, and 3# membranes (11 mm in diameter) were put in the wells of 48-well plates and the non-specimen wells were used as a normal control contrast. The fibroblast cells were seeded onto the membranes at a density of 1.0×10^5 cells/ml. The cell carrier constructs were cultured at 37°C/5% CO_2 with the medium changed every 2 days. Cell viability was determined with MTT method after cultivating for 2 and 4 days, respectively. The optical density of the medium was tested by using a RT-2100C microplate reader (Shenzhen, China) at the wavelength of 492 nm.

2.5 Evaluations of histocompatibility and degradability of membranes in vivo

The histocompatibility and degradability of membranes were examined by implanting 0# and 1# membranes into the subcutaneous tissue and skeletal muscle of Wistar rats. Twenty-one Wistar rats weighing about 200 g were kept under a specific pathogen free condition throughout the experiment. These rats were randomly divided into five groups: drug loaded membranes test group (1# group) nine, blank group (0# group) nine, and control group three.

The sterile membranes (6 mm in diameter) were implanted into the back subcutaneous tissue and leg skeletal muscle of test group and blank group rats. For the control group rats, entwisted silk suture (10 cm) was implanted. Subsequently, the pouch and the skin incision were closed. The rats implanted with medical suture were set as controls. Seven rats (test group three, blank group three, and control group one) were sacrificed on 1, 4, and 8w after the implantation, respectively. Membranes and their surrounding tissue were removed, and subsequently fixed in 4% neutrally buffered formaldehyde, embedded in paraffin, stained with haematoxylin–eosin (HE) and analyzed for histology.

2.6 Statistical analysis

Data were shown as means ± standard deviation (SD) of a representative point from parallel experiments. Statistical analysis of data was performed by one-way analysis of variance (ANOVA), and a value of $P < 0.05$ was considered significant (computed by SPSS version 13.0 Software).

3 Results

3.1 ^{1}H NMR and DS of Suc-HECTS

The ^{1}H NMR spectrum of Suc-HECTS was shown in Fig. 2. ^{1}H NMR (D_2O) δ: 1.92 (1H, s, –NH(CO)CH_3), 2.43 (4.55H, br s, –NH(CO)–CH_2–), 2.57 (1.02H, br s, –CH_2–COOH), 3.12 (1.74H, br m, H-2 of GlcN), 3.44-3.54 (br m, H-2 of *N*-acylated GlcN and H-3, H-4, H-5, H-6 of monosaccharide residue). DS was determined according to ^{1}H NMR [25, 30] and the result was 40%.

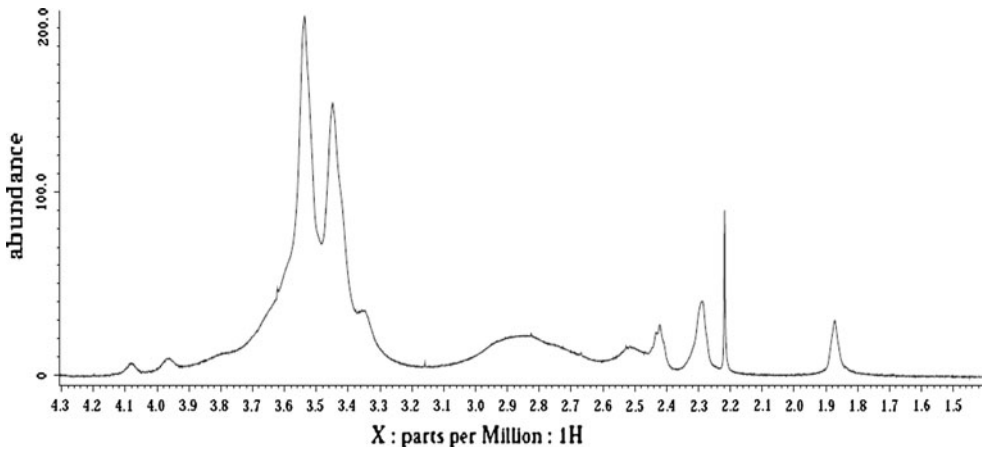

Fig. 2 ^{1}H NMR spectrum of Suc-HECTS in D_2O

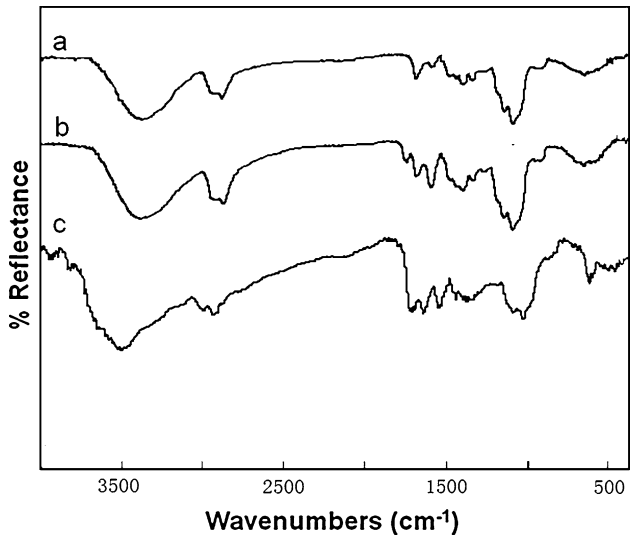

Fig. 3 FTIR spectra of (*a*) HECTS, (*b*) Suc-HECTS, and (*c*) Suc-HECTS–MMC

3.2 FTIR analysis

The IR spectra of HETCS, Suc-HECTS, and Suc-HECTS–MMC cross-linked film were presented in Fig. 3. In the spectrum of FTIR spectroscopy, for HECTS, the strong peak around 3,379 cm^{-1} indicated the stretching vibration of O–H, extension vibration of N–H, and intermolecular hydrogen bonds of polysaccharide. Distinctive absorption bands appeared at 2,919–2,876 cm^{-1} (asymmetric stretching of the–CH_2–CH_2–bridge), 1,658 cm^{-1} (Amide I) and 1,566 cm^{-1} (bending vibration of –NH_2). The peak at 1,370 cm^{-1} represented the –C–O stretching of primary alcoholic group (–CH_2–OH). The absorption bands at 1,075 and 1,059 cm^{-1}(skeletal vibration involving C–O stretching) were characteristic of its saccharine structure. Compared with HECTS, the IR spectrum of Suc-HECTS showed that the intensity of the peak at 2,925–2,872 cm^{-1} was increased and the new peak at 1,726 cm^{-1} (the –C=O stretching vibration frequency of the amide group) was formed. This confirmed that succinic anhydride was introduced into the amino groups of HECTS. The IR spectrum of Suc-HECTS–MMC showed new signals, at 3,445 and 1,742 cm^{-1} (the –C=O stretching vibration frequency of the amide group), which were attributed to the incorporation of the MMC. The peak between 3,400 and 3,300 cm^{-1} was changed, largely due to the MMC was introduced.

3.3 Equilibrium water content and swelling ratio

The swelling behaviors of CTS-based films were presented in Fig. 4. As expected, the 1#, 2#, and 3# samples showed stronger swelling property and higher water uptake than 0# sample, as a result of the predominant hydrophilic character of the carboxyl group in Suc-HECTS structure and hydrophilic drug (MMC). According to the comparison among results of 1#, 2#, and 3# samples, it was showed that the relationship between the degree of cross-linking and swelling behavior was positive. The swelling property and water absorption were directly proportional to the degree of cross-linking, which was ascribable to the interval between molecules. The molecule interval became narrow along with the increase of cross-linking density. Thus, the compacted molecular structure of membrane in high cross-linking degree made it had more swelling capability than low degree.

3.4 Permeability

The curves of NaCl, glucose and tyrosine concentration changed with time were shown in Fig. 5. Four kinds of membranes for inorganic salt, monosaccharide, and amino acid all had good permeability. 1# sample had almost the fastest penetration speed for three kinds of substances. However, the discrepancy among the remaining 0#, 2#, and 3# samples in permeability was greatly. Generally, the drug loaded membranes had better permeability than the control film.

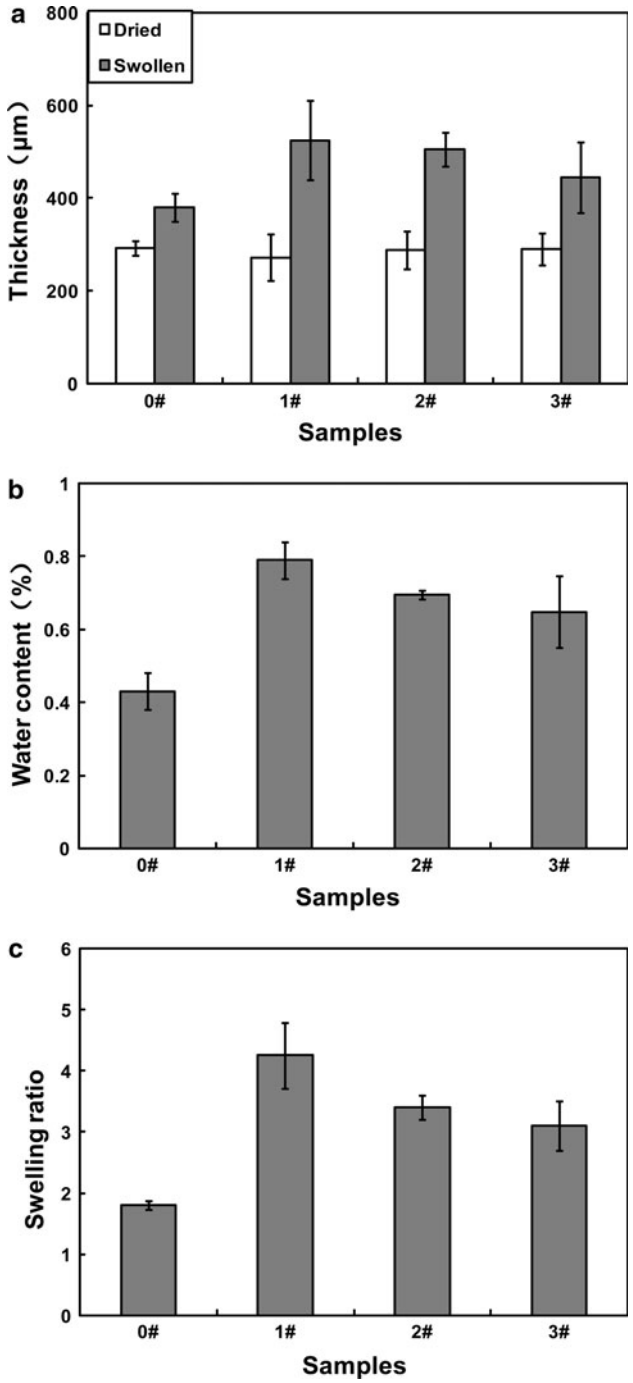

Fig. 4 Swelling behavior of samples (after 48 h in PBS): **a** is thickness of samples in dry and wet; **b** is equilibrium water content of samples; **c** is swelling ratio of samples; 0#HECTS membrane, cross-linking degree is 1:50;1#Suc-HECTS–MMC membrane, cross-linking degree is 1:20;2#Suc-HECTS–MMC membrane, cross-linking degree is 1:50;3# Suc-HECTS–MMC membrane, cross-linking degree is 1:100

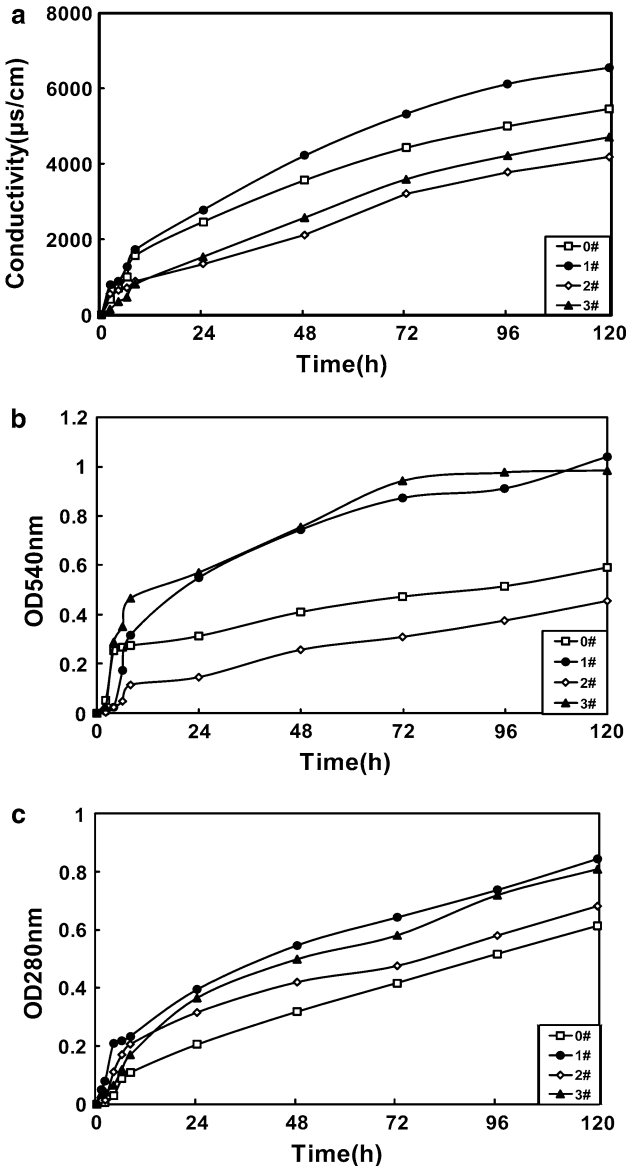

Fig. 5 Permeability properties to NaCl, glucose, and tyrosine of samples: **a** is NaCl; **b** is glucose; **c** is tyrosine; 0#HECTS membrane, cross-linking degree is 1:50; 1# Suc-HECTS–MMC membrane, cross-linking degree is 1:20; 2# Suc-HECTS–MMC membrane, cross-linking degree is 1:50; 3# Suc-HECTS–MMC membrane, cross-linking degree is 1:100

3.5 In vitro drug release study

Mitomycin C contents in the releasing medium during a test period of 360 h were presented in Fig. 6. According to this graphic, all samples showed an intensive release of MMC in the first 48 h. This behavior could be attributed to the MMC incorporated to the film surface that was easily delivered. However, as soon as the superficial MMC was released, the release rate has been gradually decreasing. The chemically combined MMC began to be released after non-bound drug molecules were almost completely released. For this reason, all samples released more than half of the theory drug-loading amount in the first 144 h. Since then, the drug release rates were more and more

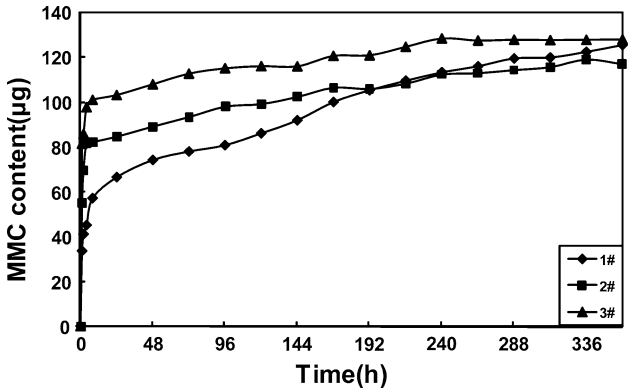

Fig. 6 Mitomycin C content in the releasing medium of samples: 1#Suc-HECTS–MMC membrane, cross-linking degree is 1:20; 2#Suc-HECTS–MMC membrane, cross-linking degree is 1:50; 3# Suc-HECTS–MMC membrane, cross-linking degree is 1:100

slowly. The cumulative releases of MMC from the three samples were 125.61, 116.90, and 128.12 μg on 360 h, which approached to the theoretical analysis (125 μg). It was considered that MMC in membranes were completely released. Furthermore, it was observed that a higher degree of cross-linking produced a lower release, which was thought to relate to the fact that cross-linking creates a polymeric network that might act as a barrier to prevent diffusion of MMC. Comparing samples with the same MMC initial concentration but different degrees of cross-linking (1#, 2#, and 3#), it was shown that a higher degree of cross-linking promoted slower percent release and a lower degree of cross-linking showed a higher percent release. Thus, the diffusion rate was inversely proportional to the degree of cross-linking. The mechanism of MMC diffusion was mainly governed by the cross-linking density.

3.6 Activity of L929 fibroblast on membranes

To investigate the fibroblast proliferation activity on membranes, we cultured the L929 fibroblast cells on the membrane. Cell viability at different time intervals represents the active mitochondrial enzymes present in a cell capable of reducing MTT. The L929 fibroblast proliferation studied by MTT assay was showed in Fig. 7a. As shown, $OD_{492\ nm}$ were significantly lower when the fibroblast cells were cultured on the drug loaded membranes than they were when cultured on the control ($P < 0.05$). The results demonstrated that the drug loaded membranes could inhibit L929 fibroblast cell growth and proliferation.

The relative growth rate (RGR, %) of 0#, 1#, 2#, and 3# samples were demonstrated in Fig. 7b. The MTT results were further analyzed to inspect the fibroblast suppression of 1#, 2#, and 3# membranes. The percentage of viable L929 cells in 0# group was around 90% compared with the control group, and the cytotoxicity was level 1. Thus, 0# membrane was no effect on the fibroblast survival. However, the viability of L929 cells obviously decreased in contact with 1#, 2#, and 3# drug loaded membranes prepared with different cross-linking degrees. They had significant difference among the drug loaded groups. The experimental conclusion corresponded with the result of morphological observation.

3.7 Histocompatibility and biodegradability in vivo

No observable signs of inflammation, infection or distress were noticed in any of the animals implanted with the four kinds of membrane for 2 months. Inflammatory reaction was serious in the suture experiment group during the observation period. The membrane of 1# group was not

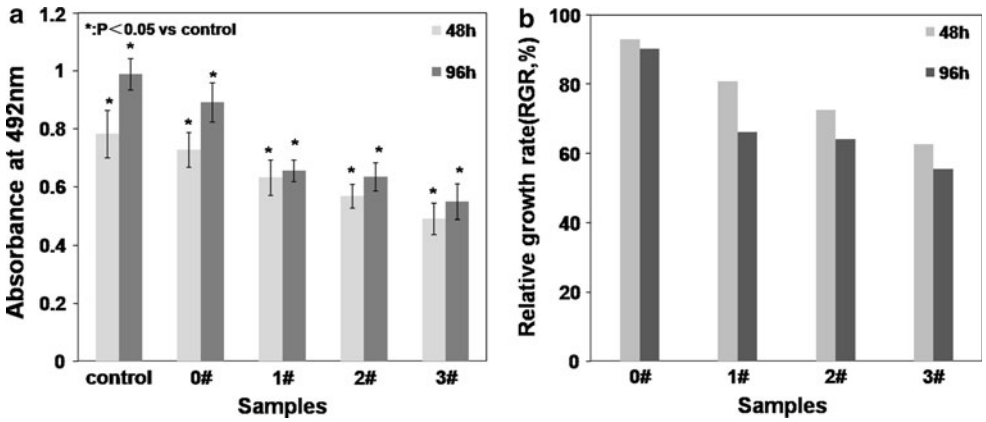

Fig. 7 Analysis of the $OD_{492\ nm}$ values at 48 and 96 h for L929 fibroblasts under different conditions. **a** MTT assay for cell viability was carried out for L929 cells seeded on 0#, 1#, 2#, 3#, and control at 48 and 96 h; **b**Relative growth rate (*RGR*) of different samples: control with non-membrane;0#HECTS membrane cross-linked ratio of 1:50;1# Suc-HECTS–MMC membrane cross-linked ratio of 1:20;2#Suc-HECTS–MMC membrane cross-linked ratio of 1:50;3# Suc-HECTS–MMC membrane cross-linked ratio of 1:100

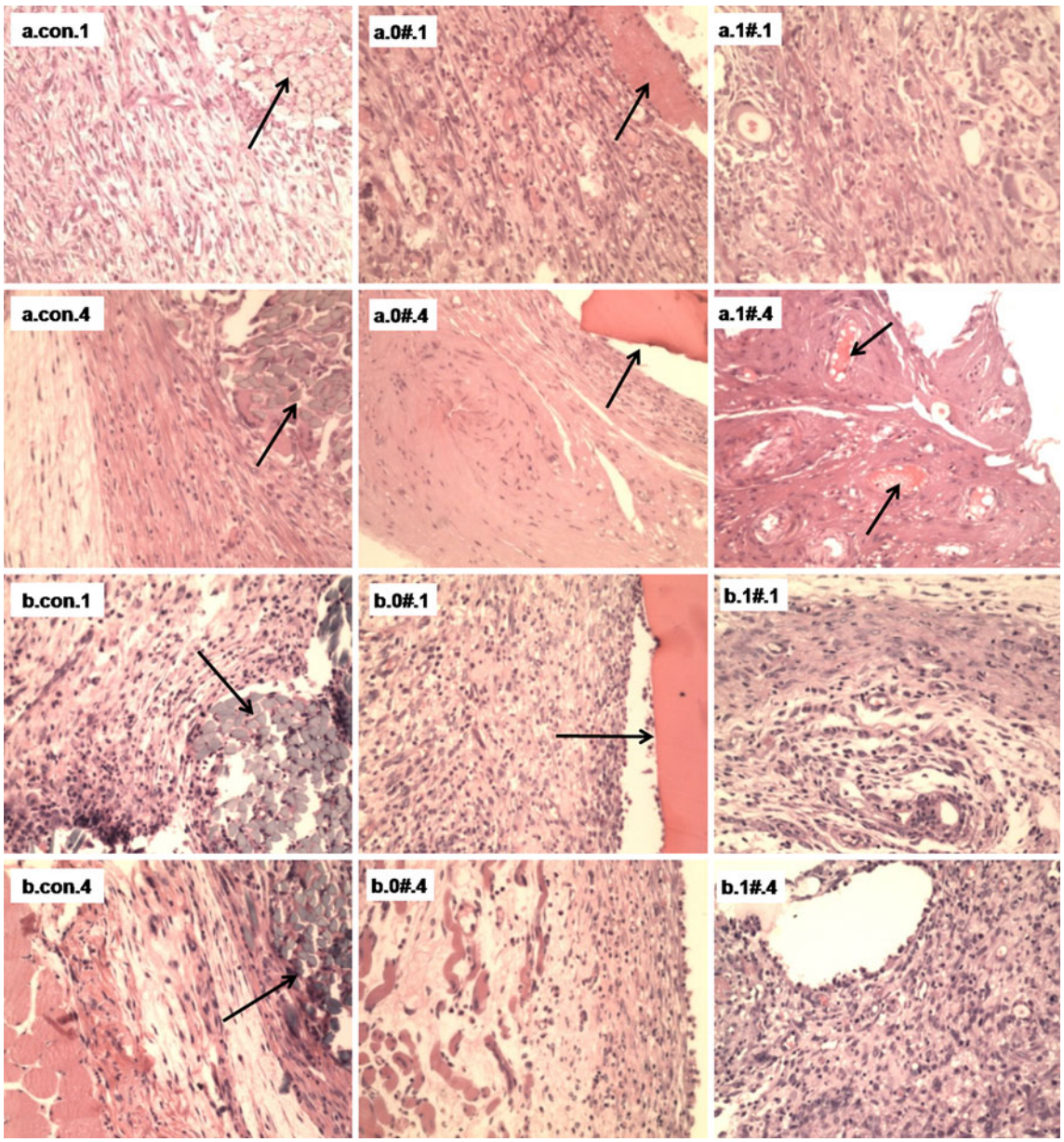

Fig. 8 Photomicrographs of the tissues implanted with: suture (*con.*) and blend membranes (0#0.1#.) were retrieved after 1 and 4 weeks (1.4.). The subcutaneous (**a**) and muscle (**b**) tissue were stained with HE (original magnification ×200). Note sutures and membrane fragments (*arrows*) in graphs. Inflammatory reaction was serious in the suture experiment group (*con.*).The encapsulation formation of 0# implants was observed during the trial period

significantly changed in the first 4 weeks and thoroughly degraded after 8 weeks, except encapsulation was observed in week one. In the whole observation, the 0# sample had no obvious degrading phenomena.

However, post-mortem examination by pathological section detected some inflammations of the implant encapsulation at the interface between the material and the tissue in tested animals. In week one, similar inflammations were observed in both membrane groups (Fig. 8a, b.0#.1#0.1). In week four, inflammation of the 0# and 1# experimental group were significantly reduced. The tissues of 0# study group had obvious fibrous encapsulation surrounding the implanted membrane (Fig. 8a, b.0#0.4). In this time, the 1# implants in hypodermic and muscle were partial degraded (Fig. 8a, b.1#0.4). On week eight post-operatively, the drug loaded membranes were totally degraded into fragments with light inflammatory responses around the implants and their surrounding areas. The 0# membranes had no evident change at 8 weeks and generated encapsulation of dense connective tissue, but slight inflammation was showed surrounding encapsulation and implant. In contrast, animals of control group displayed

significant inflammatory responses in the tissue around sutures during the observation period (Fig. 8a, b.).

4 Discussion

The ideal surgical procedure for the treatment of glaucoma is one which is technically easy to perform, effectively, and consistently reduces IOP, low risk of hypotony and related complications. Many of the most significant risks arise because of the formation of a nonphysiologic bleb, and as a result, many of the innovations aim to avoid the formation of such a subconjunctival reservoir [31]. The development of surgical devices continues to improve the safety of glaucoma surgery and to lower risk profiles. The study on the applications of antifibrotic drug makes inhibiting the formation of cicatrization and avoiding the complication possible. It would be ideal to inhibit the fibroblast proliferation on the filter surgery without influencing the reproductivity and metabolic activity of other type of cells.

The present research mainly includes two aspects. One is testing medication that may inhibit scar formation and have less cytotoxicity [32], and the other is studying and designing biomedical materials in order to meet the demands of decreasing the IOP and preventing postoperative adhesion after trabeculectomy [33, 34].The combination of antifibrotic drug and carrier of drug delivery system may be the future trend of glaucoma filtering surgery development.

The excellent chemical and biological properties of CTS make this natural polymer as an ideal candidate for controlled drug release formulations [35, 36]. HECTS as a kind of CTS derivatives exhibits good solubility and biocompatibility. Suc-HECTS is another kind of CTS derivatives, which is obtained by chemical modification of HECTS. Because of the presence of carboxyl, Suc-HECTS is better than HECTS in terms of hydrophilicity and biocompatibility. Therefore, Suc-HECTS is more suitable as a drug delivery carrier. Due to the poor mechanical strength of the Suc-HECTS membrane and HECTS membrane in water, both membranes need to be cross-linked as a drug delivery carrier.

The conjugate prepared by the direct coupling of MMC with Suc-HECTS using EDC·HCl was obtained due to cross-linking among the polymer supports [26, 27]. A secondary amino group of MMC was utilized in the conjugation reaction. In the process, another compound is produced because of cross-linking within or among the polymer supports. Since the cross-linking was considered to be formed between amino groups and carboxyl groups of Suc-HECTS, increased *N*-succinylation of Suc-HECTS was expected to reduce the undesired cross-linking. Moreover, a small amount of EDC·HCl was employed for the purpose of the suppression of the condensation reaction between amino groups and carboxyl groups of Suc-HECTS. In addition, a reduction of the reaction time was also applied to suppress the cross-linking.

The DS of Suc-HECTS was calculated by ^{1}H NMR (Fig. 2). The special structures in HECTS, Suc-HECTS, and Suc-HECTS–MMC were tested by using FTIR. Through comparative analyzes (Fig. 3) we found that HECTS was successfully combined with succinic anhydride and MMC successively.

The swelling behavior is also one of important aspects to be investigated for drug loaded membrane. The retention and the release of drug molecules depend on the swelling property of membrane, which may vary by several factors including the amount of water in membrane and swelling ratio. The moderate hydrophilicity of the membrane will ensure the membrane be thoroughly degraded after the wound healed [19].The water content of medicine-carried membrane was effected by several parameters, including the hydrophilicity, stiffness, and pore structure of a matrix. The swelling extent of the drug loaded group was higher than the sample of HECTS without drug. The comparison results suggested that the more hydrophilic group of Suc-HECTS–MMC might be the major factor that influences the swelling degree of these samples. Through comparison among the swelling results of drug loaded samples, it could be found that swelling behavior was directly proportional to the density of cross-linking. The higher cross-linking degree it had, the higher equilibrium water content and swelling ratio it showed. The moderate degree of hydroscopicity of membranes provided excellent flexibility and mechanical property.

Permeability of glucose and other small molecules is an important determinant to success for drug loaded membrane in glaucoma filtering surgery. By selecting an appropriate permeability of the membrane it may be possible to improve intraocular aqueous flow and decrease the IOP. In addition, it could ensure the nutrition in the aqueous fluid transfers into the stroma and epithelium tissue. The experimental results (Fig. 5) demonstrate the good permeability of the Suc-HECTS drug loaded membrane.

The release of MMC from the drug loaded membrane in vitro is shown in Fig. 6. The release rate depended on the cross-linking density, increasing with the low degree of cross-linking. It may be proposed as an interpretation that the drug molecules are not only bound to Suc-HECTS but also encapsulated in the membranes due to tight cross-linking among the Suc-HECTS molecules. The compacted molecule structure of Suc-HECTS–MMC created a barrier between drug and surroundings.

The effect of drug loaded membrane on proliferation of L929 fibroblasts in our study was researched. The MTT

results showed that the effects were statistically significant a biologically relevant decrease in cell migration (Fig. 7).The control group of HECTS (0#) was observed slightly inhibiting function. It was considered that the HECTS membrane had smooth surface to lead to inhibition of fibroblast cell attachment. The experimental group of drug loaded (1#, 2#, and 3#) had the same initial concentration of MMC in theory. The antifibrotic drug (MMC) concentration of culture medium in each well was only effected by the rate of drug release in vitro. The analysis results of MTT were supported by the curves of MMC release in vitro (Fig. 6).

Histocompatibility and biodegradability of the membranes were evaluated in a rat model via the subcutaneous and intramuscular implantation. It has been reported that when a biomaterial was implanted, the local tissue reacted initially to the injury, and then to the presence of the material. Inflammation is the most common reaction to all injury forms. And the inflammation normally leads to repair of the affected tissue. However, abnormal wound healing can result in damage to the normal tissue [37, 38]. In this study, no serious inflammation was observed during the experimental period with the naked eye. Pathological sections showed that all membranes resulted in an acute inflammation reaction within the first 4 weeks post implantation. But the drug loaded membrane had a higher rate of degradation and a reduced inflammatory response than blank membrane at the later stage. Compared with the suture control group, the degrees of inflammatory reactions in surrounding tissue implanted with the membranes were reduced in the whole experimental period (Fig. 8). The difference of biodegradation between drug loaded and non-drug samples was partly caused by the different hydrophilicity of HECTS and Suc-HECTS–MMC molecular structure. On the other hand, the pore structure posed by the MMC release of biodegradable membranes had a significant effect on the degradation speed of implants. It was assumed that non-bound MMC molecules played an important role as the pore-forming agent.

5 Conclusions

In this study the advantages of the antifibrosis drug (MMC) and CTS derivative (Suc-HECTS) was successfully combined via preparation of a porous drug loaded membrane. The theory and experimental results indicated that the novel membrane was effective on the swelling property, permeability, and drug release in vitro. These features might be explained by the molecular structure of Suc-HECTS–MMC and the cross-linking among the Suc-HECTS particles in the membrane. The excellent performance of this membrane in suppression of fibroblast proliferation and histocompatibility, biodegradability in vivo demonstrated the potential use of the membrane as a new biomaterial for glaucoma filtering surgery. All experiment results provided a theoretical basis for the future development of drug loaded implant material based on CTS for glaucoma treatment.

They also pointed out the further research direction of the Suc-HECTS–MMC material as a possible implant for trabeculectomy. The future work is concentrated on two directions:

(1) the period of degradation in vivo must be improved;
(2) animal experiments such as the rabbit glaucoma filtering surgery by estimating the variation of IOP and the filtering bleb are necessary to evaluate the antifibrosis effect of this drug-carrier membrane in vivo.

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