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Biocompatibility evaluation of N,O-hexanoyl chitosan as a biodegradable hydrophobic polycation for controlled drug release

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Abstract N,O-hexanoyl chitosan (HC) displayed unique properties of excellent processibility, solubility in common organic solvents and cationic characteristics, thereby might find versatile applicaions for biomedical engineering. In this study, the biocompatibility of HC was evaluated. The effect of HC on dermal fibroblast attachment, proliferation and viability was examined in vitro, in comparison with poly(lactide-co-glycolide) (PLGA) and chitosan. The in vivo tissue response to HC was compared with PLGA implanted subcutaneously; the weight loss of the implant materials was also measured duing the degradation process. It was found that HC had no deleterious effect on the viability of dermal fibroblast. In vivo, HC displayed a favorable tissue response profile compared with PLGA, with significantly less inflammation and fibrosis. The erosion rate of HC could be modulated by changing the degree of substitution of hexanoyl groups in HC.

1 Introduction

Biodegradable polymers were widely studied and used for various biomedical applications including controlled drug release and tissue engineering, which could be classified as hydrophobic polymers and water-soluble polymers according to their solubility [1]. In general, hydrophobic polymers, such as PLGA and polyanhydrides, always have excellent processing properties and lack of functional groups for modification [2, 3]. Although tremendous works have been done for functionalization of hydrophobic biodegradable polymers [4–6], the synthetic scheme was always very tedious and time-consuming. On the contrary, most of water-soluble polymers, such as chitosan, carry several functional groups for derivation. However, it was always necessary to crosslink the polymers for bio-related applications and most of crosslinking agents were toxic [7]. Therefore, the materials combining the advantages of the hydrophobic polymers and water-soluble macromolecules to circumvent the shortcomings of the individual polymers would find versatile applications for biomedical engineering.

Chitosan is a unique cationic polysaccharide, which is mainly composed of β-(1→4)-2-amino-2-deoxy-D-glucopyranose residues [8]. Up to date, chitosan has been widely studied for a variety of biomedical applications including controlled release, tissue engineering, gene delivery and wound dressing etc., due to its natural-abundant resource, unique cationic nature, biocompatibility and biodegradability [9–12]. However, the poor solubility of chitosan in either water or organic solvents limits its applications in these fields. Numerous modification strategies have been proposed to improve its solubility and widen its applications [13–15]. For example, N,O-acylated chitosan can be conveniently prepared by acylation reaction using $MeSO_3H$ as a solvent [15, 16]. The resultant chitosan derivative can be soluble in dimethylformamide (DMF) or chloroform. In addition, acylation of amino groups carried by chitosan can be partially avoided without the need of

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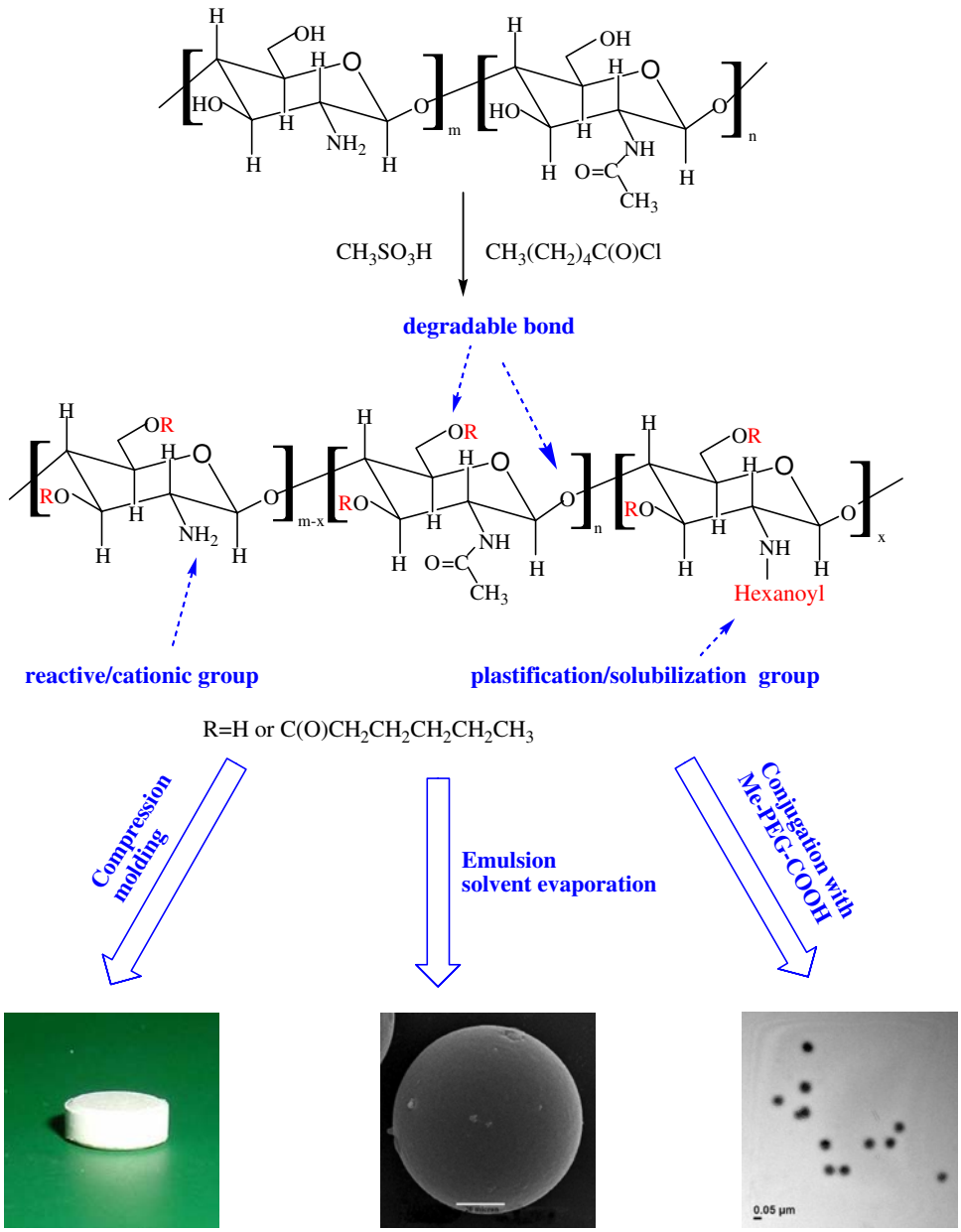

Fig. 1 Synthetic scheme of HC

any protection–deprotection processes. We demonstrated the semi-synthetic polymers were versatile for controlled drug release (Fig. 1). By regular compression molding at room temperature, tablets could be prepared with the sustained release capacity of ibuprofen for more than 3 days. Microspheres composed of hexanoyl chitosan could be formulated by oil-in-water emulsion solvent evaporation method. Furthermore, pH-sensitive micelles with average diameter below 100 nm could be assembled from the copolymers of PEGylated hexanoyl chitosan [16]. It was also reported that nanofibrous composite scaffolds could be prepared by co-electrospinning of PLGA and hexanoyl chitosan, which may find applications for tissue engineering [17]. Although hexanoyl chitosan was approved to be a versatile biomaterial for various applications, it is essential to verify its biocompatibility before it could be used for biomedical engineering. In this manuscript, the biocompatibility of hexanoyl chitosan was evaluated in vitro and in vivo. In addition, the in vivo degradation behavior of HC was also studied.

2 Experimental details

2.1 Materials

Hexanoyl chitosan was synthesized according to the scheme previously reported [15]. The substitution degree

Table 1 Characteristics of hexanoyl chitosan

Polymer	HC-2	HC-3	HC-4	HC-6
DS	0.62	1.14	1.26	1.56

of hexanoyl groups in the chitosan derivative was listed in Table 1. Poly(lactide-co-glycolide) (PLGA) was purchased from Birmingham (Birmingham Polymers, Birmingham, AL, USA).

2.2 Polymer fabrication

Cylinder-shaped samples of about 100 mg of HC, 2.8 mm in diameter and approximately 9.6 mm in length, were prepared by compression molding with a homemade apparatus at 100 kg/cm^2 and 37°C. Polymer cylinders were exposed to UV radiation for 60 min on each side to minimize bacterial contamination.

2.3 In vitro evaluations of HC disks

HC disks and control membranes were rinsed extensively in sterile ddH_2O and PBS, respectively, before transferring to cell culture medium. Samples (n = 3) were co-cultured with approximately 200 µl (1×10^4 cells/ml) of mouse dermal fibroblasts (CRL-2017, passage 2–20, ATCC, Manassas, VA, USA) in DMEM (Gibco, Grand Island, NY, USA) supplemented with 10% Fetal Bovine Serum (Hyclone, Logan, UT, USA) and 1% Penicillin/Streptomycin solution (Gibco, Grand Island, NY, USA) at 37°C under a humidified atmosphere of 5% CO_2. Monolayer cells maintained under comparable conditions were used as controls. Chitosan membrane and PLGA film were set up as benchmark biocompatible materials for reference. The culture media were changed daily; cell morphology, adhesion, viability and proliferation were observed under an inverted phase contrast light microscope (Axiovert 200M, Zeiss, Munich, Germany). Images were acquired with Axiovision 4 imaging software (Zeiss, Jena, Germany).

Cell viability studies were performed using MTS assay (CellTiter 96® aqueous non-radioactive cell proliferation assay kit, Promega, Madison, WI, USA) to verify the biocompatibility of both disks and their degradation byproducts. Samples were taken at day 1, 3, 5 and 7, respectively. Likewise, Live/Dead staining assay (Molecular Probes, Eugene, OR, USA) were utilized to corroborate the results obtained by MTS assays. At day 7 after seeding, a fresh piece of cell-laden disk was moved to a fresh cell culture well and rinsed with PBS twice, followed by incubating in 200 µl Live/Dead dye solution for 10 min and then observed under a fluorescent microscope.

2.4 Implant test

Animals were anesthetized by intraperitoneal injection with a mixture of ketamine HCl (87 mg/kg body wt.) and xylazine (13 mg/kg body wt.). The rats were shaved and sterile-prepped with Betadine and isopropyl alcohol. An incision (approximately 1.0 cm long) was made laterally on the dorsum using a surgical blade. Subcutaneous pouches were made away from the incision using blunt scissors, the HC cylinders were inserted and the wounds closed using surgical suture.

The animals were sacrificed by CO_2 asphyxiation sequentially at specific time points after surgery. The polymers were taken out for characterization by weight loss, IR and SEM [13]. The tissues adjacent to the matrices were harvested using a surgical blade and placed in 10% formalin solution for at least 7 days. The fixed tissues were trimmed, paraffin-embedded, sectioned (4–5 µm thick) with a microtome and stained with hematoxylin and eosin. Tissue samples were examined by light microscopy.

2.5 Statistical analysis

At each time point, five replicates were tested for HC and control samples. The results were expressed as mean values with standard deviations. A Mann–Whitney U test was used to statistically compare experimental values at a given time point; a $P < 0.05$ was considered statistically significant.

3 Results and discussions

3.1 In vitro study

MTS assay were performed to quantify the living cells and thus the viability of cells under the influence of the disks and the results are depicted in Fig. 2. Considerable elevation of cell numbers on all samples co-cultured with disks as well as controls one day after cell seeding were observed. Cells in all samples continued to proliferate and reached full confluence at 5 days after seeding. Further incubation (i.e., 7 days) did not result in appreciable change in cell growth. The viabilities of cells in the presence of disks were comparable to those of controls and those incubated with benchmark reference biocompatible biomaterials as well. Cell numbers on the discs of HC-3 and HC-4 were lower than those on other HC discs,

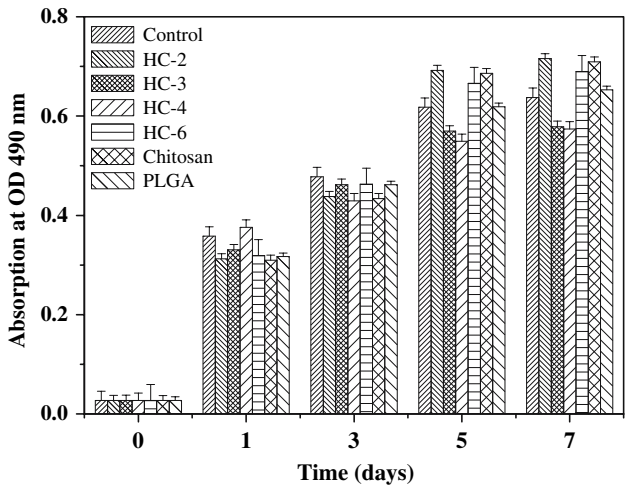

Fig. 2 MTS assay for evaluation of cell proliferation and viability in the presence of HC disks compared with monolayer controls and reference biomaterials

chitosan and PLGA. The reasons were still unclear. Overall, the results suggested that the disks did not affect normal cell proliferation and viability; therefore they were non-toxic.

As depicted in Fig. 3 Live/Dead staining of cells attached on the disks confirmed cell viability of cells when in direct contact with the disks. Apparently, cells attached to the surface of the disks and their general morphologies were comparable to the monolayer controls. On the contrary, cells did not attach to chitosan membrane and PLGA film. Seven days after seeding on disk surfaces, cells were highly compact and formed multi-layers on the disk surfaces (Fig. 3a, see disk edge). Figure 3b illustrated that virtually all cells on the disk surface were viable. There was no noticeable difference between various disk formulations in terms of cell viability (data not shown).

3.2 In vivo study

The weight loss profiles of the modified chitosan with different compositions were displayed in Fig. 4, which verifies the biodegradability of hexanoyl chitosan. The erosion rate of the materials could be modulated by the degree of substitution of hexanoyl groups (DS) in HC. With the increase of DS, there was a slight acceleration in the weigh loss of the modified chitosan. For example, the weight loss of HC-6 was about 32.36% on days 35 after degradation, which decreased to 24% for HC-2. The changes in the chemical structure of HC during degradation were monitored with FTIR. The results were shown in Fig. 5. On days 62 after in vivo dgradation, the absorption intensity of ester groups at 1,720 cm^{-1} became very low, which indicated that the erosion of the hydrophobilized chitosan mainly resulted from the cleavage of ester bonds between hexanoyl groups and chitosan backbone. This is the reason why the erosion rate of HC increases with DS of

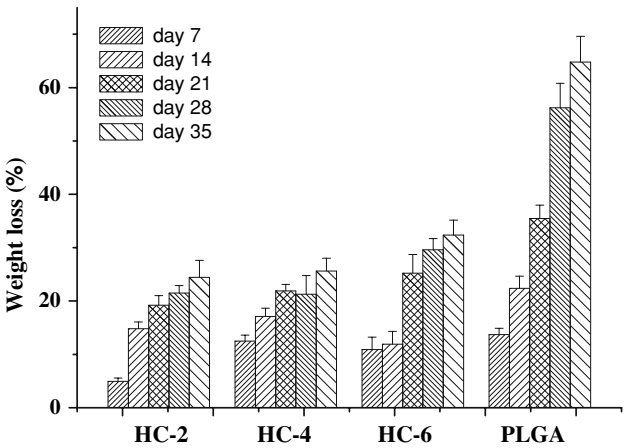

Fig. 4 Weight loss of HC implanted subcutaneously

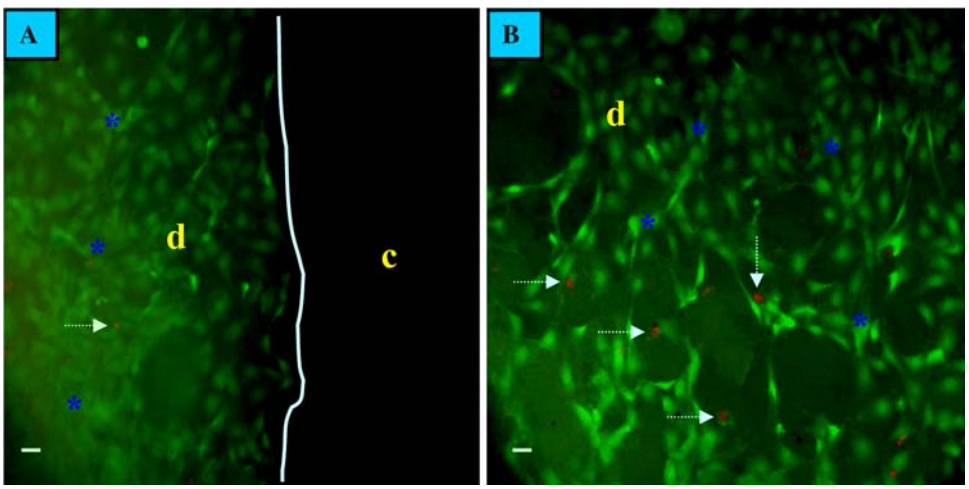

Fig. 3 Viability of cells on HC disk surface. (**a**–**b**) Samples were stained with Live/Dead staining kit. (**a**) The edge of disk (outlined with white line); d: disk; c: cell culture well; Star: living cells (green); arrow: dead cells (red); (**b**) The surface of disk. Scale bars: 20 μm

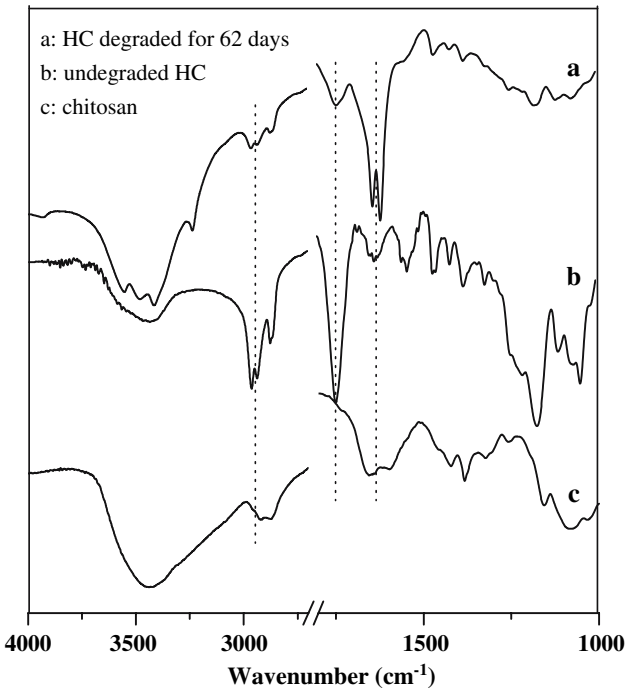

Fig. 5 IR spectra of chitosan, undegraded HC and HC degraded for 62 days

hexanoyl groups. Compared with PLGA, HC undergoes much slower erosion, possibly resulting from the higher hydrophobic nature of ester groups in HC. It was found that the degraded samples maintained their shapes over the evaluated period of time. However, the solubility of the samples in DMF gradually lost with degradation, which can be attributed to a decrease in the content of hexanoyl groups in the degraded samples. On days 28 after surgery, the PLGA samples shrunk significantly.

During the in vivo assessment, there were no behavior changes or visible signs of physical impairment indicating systemic or neurological toxicity observed. Gross observation of the implant sites revealed implants surrounded by a fibrous capsule for all the modified chitosan. The tissues adjacent to the implants were taken for analysis by light microscopy. The inflammatory reactions were analyzed quantitatively.

In general, HC and PLGA induced different early inflammatory response, as indicated by recruitment of lyphocytes and granulocytes + macrophages, and by fibrotic band thickness. A significant acute inflammatory component of neutrophils was not detected. As shown in Figs. 6a and 7, similar numbers of lymphocytes were observed on days 7 in tissue implanted with HC and PLGA, although the averages were higher in the HC samples in comparison with PLGA. However, the lymphocyte population on days 35 was significantly lower in HC samples than in PLGA samples and was comparable to that in the sham surgery samples (Figs. 6a and 8). The response of

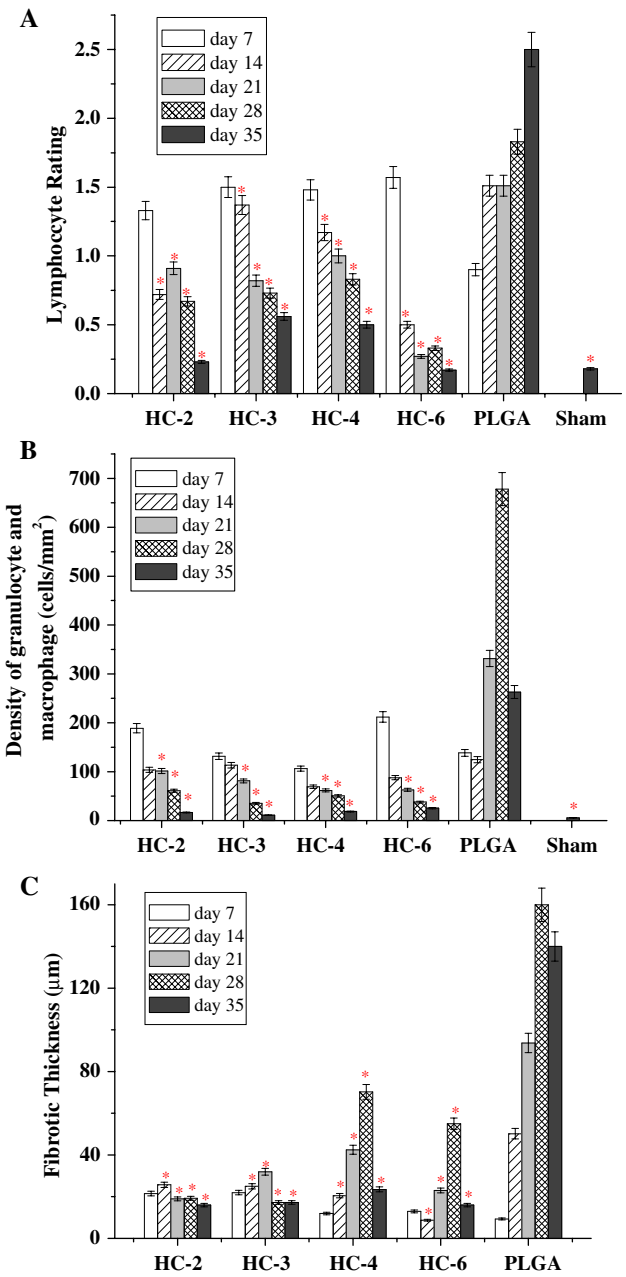

Fig. 6 Inflammatory responses to HC

granulocytes and macrophages to HC implantation was similar to that of lymphocytes (Figs. 6b and 8).

The fibrous capsule thickness was observed to be different between the HC and PLGA samples. As with the inflammation measures, HC, PLGA, and sham surgery samples had similar fibrous capsule thicknesses on days 7 (Figs. 6c and 7). However, the observed thicknesses sharply diverged on days 14. The fibrous capsule thicknesses decreased in HC and sham surgery samples at the remaining harvest points, while the thickness increased significantly on days 21 and 35 in the PLGA samples.

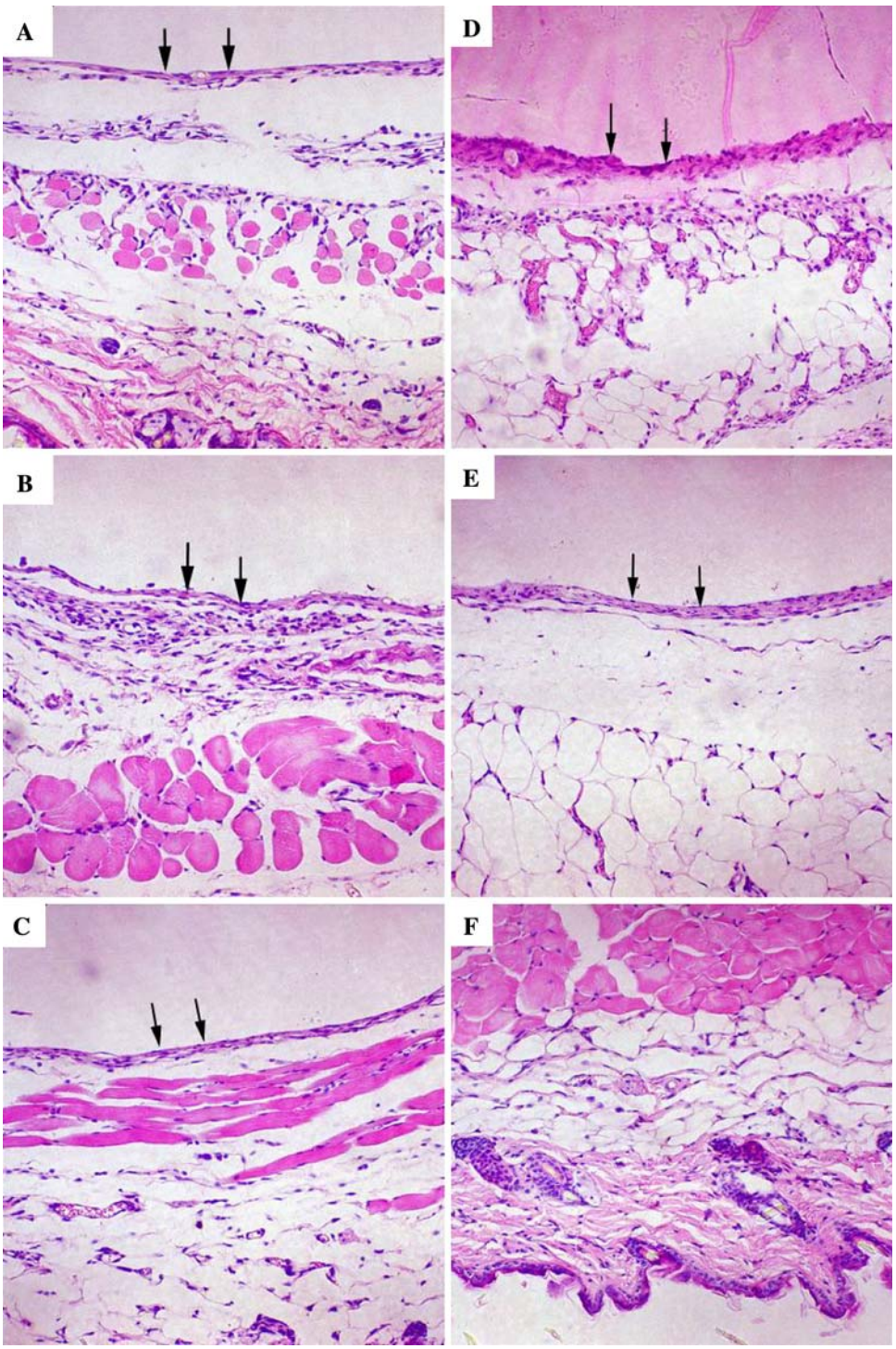

Fig. 7 Micrographs of rat subcutaneous tissues implanted with hexanoyl chitosan on day 7. (**a**) HC-2, (**b**) HC-3, (**c**) HC-4, (**d**) HC-6, (**e**) PLGA, (**f**) sham. 150×

The lymphocytic and fibrotic reactions most likely resulted from the polymer degradation behavior. As indicated from the erosion data (Fig. 4), mass was slowly but consistently lost from the HC samples. Correspondingly, the changes in lymphocytic infiltrate level and the fibrous capsule thickness were not significant throughout the HC implantation period. In contrast, PLGA underwent an initial period of water uptake with significant swelling, followed by a period of rapid mass loss by day 21. This sudden mass loss caused a tissue response spike, as both

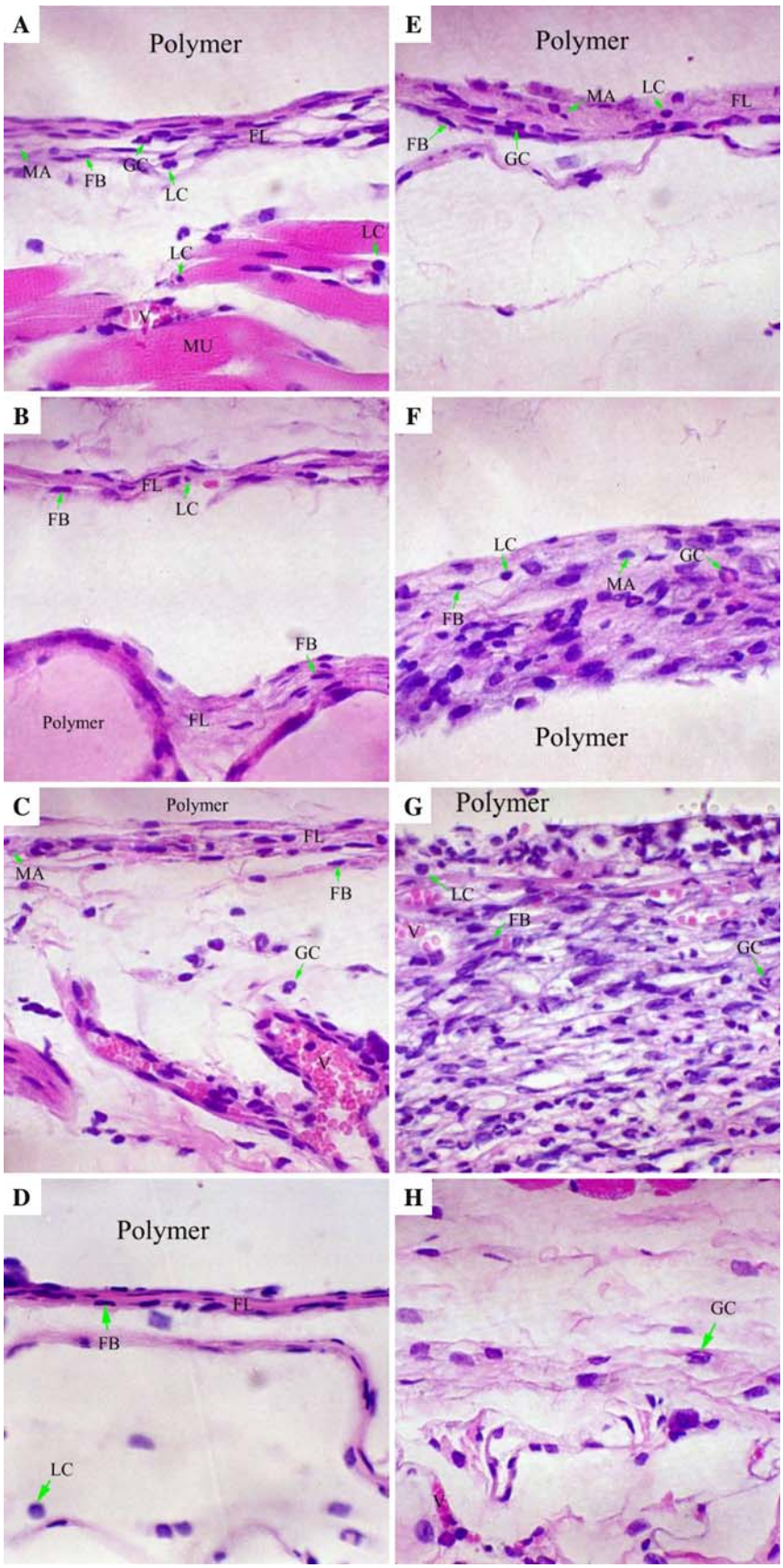

Fig. 8 Micrographs of rat subcutaneous tissues implanted with (**a**–**d**) HC-4, day 7, 14, 28, 35; (**e**–**g**) PLGA, day 7, 14, 28; (**h**) sham. 600×

the lymphocytic infiltrate level and the fibrotic zone thickness increased significantly.

4 Conclusions

The proliferation and viability of dermal fibroblast were not affected by the presence of hexanoyl chitosan. The modified chitosan could be degraded in vivo and the erosion rate could be modulated by changing the DS of hexanoyl groups. The inflammatory response to HC implanted subcutaneously was mild and lower than that to PLGA. The low cytotoxicity and good histocompatibilty of HC, as well as its unique properties of processibility and cationic characteristic, make it promising for biomedical engineering.

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